NANOTUBES DE CARBONE MONO-PAROIS PRODUITS PAR PLASMA THERMIQUE
INDUCTIF:
ÉVALUATION DE LA CYTOTOXICITÉ DES MATIÈRES PREMIÈRES ET DU
PRODUIT FINAL POUR UNE APPLICATION OSSEUSE POTENTIELLE

SINGLE-WALLED CARBON NANOTUBES PRODUCED BY INDUCTION THERMAL
PLASMA:
CYTOTOXICITY EVALUATION OF THE FEEDSTOCK MATERIALS AND THE FINAL
PRODUCT FOR A POTENTIAL BONE APPLICATION

Thèse de doctorat
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Love is a better teacher than duty

Albert Einstein
RÉSUMÉ

L'un des problèmes les plus difficiles auxquels les technologies liées aux nanomatériaux font face est l'impact qu'elles ont sur la santé humaine et l'environnement. Il est donc primordial d'étudier les effets toxicologiques de ces technologies dont l'utilisation est très répandue dans divers domaines d'application. Par conséquence, dans ce projet, la cytotoxicité des matériaux présents dans la synthèse de nanotubes de carbone mono-parois (SWCNTs) par plasma thermique inductif (des matières premières au produit final) a été évaluée.

Tout d'abord, l'influence du procédé plasma thermique inductif sur les propriétés physico-chimiques et cytotoxiques des matières premières (les catalyseurs commerciaux Co, Ni, Y2O3, Mo et le noir de carbone) a été déterminée. Un effet cytotoxique plus important a ainsi été révélé pour le Co commercial. De plus, bien que le procédé de plasma affecte les propriétés physico-chimiques de chaque catalyseur, seule la cytotoxicité du Ni a augmenté. La comparaison des particules de Ni après traitement par plasma avec les nanoparticules de Ni commerciales, a révélé que ces particules ayant pourtant une surface similaire avaient des cytotoxicités différentes. De plus, la toxicité des catalyseurs n'était pas principalement due à la libération d'ions.

Afin d'évaluer la capacité du procédé de plasma thermique inductif à synthétiser des SWCNTs de haute qualité en utilisant des catalyseurs non toxiques, les effets du type et de la quantité de trois mélanges de catalyseurs (Ni-Y2O3, Ni-Co-Y2O3, et Ni-Mo-Y2O3) sur la production de SWCNTs ont été examinés. Les calculs thermodynamiques, en phase gazeuse et dans la phase de solution liquide, ont également été réalisés. Les résultats ont montré que le type de catalyseur affecte la qualité des SWCNTs et une qualité similaire peut être produite lorsque la même quantité de Co a été remplacée par le Ni.

L'influence des SWCNTs produits avec trois mélanges de catalyseurs sur le comportement des préostéoblastes murins MC3T3-E1 a été évaluée. Les SWCNTs ont été ajoutés sur les cellules attachées ou les cellules ont été ensemencées sur des plaques recouvertes de SWCNTs. Les SWCNTs ajoutés sur les cellules attachées affectent considérablement la viabilité cellulaire. Toutefois, la viabilité des cellules ensemencées sur les SWCNTs a seulement légèrement diminué à 24 h, même pour celles ensemencées sur les SWCNTs produits avec Ni-Co-Y2O3. De plus, les cellules peuvent proliférer en présence des SWCNTs dans les 48 h. Ainsi, sauf perturbation mécanique membranaire, ces SWCNTs ne semblent pas induire de cytotoxicité sévère sur les préostéoblastes. Les SWCNTs ont donc été purifiés et leur influence sur la prolifération des préostéoblastes, l'activation de la voie des BMPs ou Smad et la différenciation ostéoblastiques induites par l'addition de BMP-2 et BMP-9 a été étudiée. Le prétraitement des cellules par des SWCNTs pendant 24 h a accéléré l'activation des Smad1/5/8 induite par les BMPs. Après 72 h d'incubation avec BMP-2 ou BMP-9, les préostéoblastes prétraités avec des SWCNTs exprimaient des gènes codant pour des marqueurs ostéogéniques comme ostérix et octéocalcine et présentaient une forte activité de la phosphatase alcaline. Fait intéressant, la BMP-9 a favorisé la différenciation des préostéoblastes prétraités avec les SWCNTs de manière plus importante que la BMP-2. Par conséquent, la combinaison de la BMP-9 avec les SWCNTs semble être une voie prometteuse pour la régénération osseuse.

Mots-clés : Nanotubes de carbone, nanoparticules métalliques, plasma thermique inductif, cytotoxicité, prolifération cellulaire, activités enzymatiques mitochondriales, lactate déshydrogénase, ostéogénèse.
ABSTRACT

One of the most challenging issues that the technologies related to nanomaterials face is the impact they have on human health and environment. It is therefore of great importance to investigate the toxicological impacts of these technologies prior to their widespread utilization in different fields of application. Therefore, in this study, the cytotoxicity of the materials present throughout the process of single-walled carbon nanotubes (SWCNTs) synthesis by induction thermal plasma (from the feedstock materials to the final product) was evaluated.

First of all, the influence of the induction thermal plasma process on the physico-chemical and cytotoxic properties of feedstock materials (i.e. commercial Co, Ni, Y₂O₃, Mo catalysts and carbon black) was investigated. The strongest cytotoxicity was observed for commercial Co compared to other catalysts. Although the thermal plasma process affected the properties of all catalysts, only the cytotoxicity of Ni was increased. Comparing the properties and cytotoxicity of the plasma treated Ni particles with commercial Ni nanoparticles revealed that the particles with similar surface area had different cytotoxicities. Plus, the observed cytotoxicity of the catalysts was not mainly due to the release of ions.

In order to evaluate the capacity of the RF induction thermal plasma process to produce high quality SWCNTs using non-toxic catalysts, the effects of the type and quantity of three catalyst mixtures (Ni-Y₂O₃, Ni-Co-Y₂O₃, and Ni-Mo-Y₂O₃) on SWCNTs synthesis were examined. Thermodynamic calculations, in gas and particularly in liquid solution phases, were also performed. The results showed that catalyst type affected the quality of the SWCNT final product and similar quality SWCNTs was produced when the same amount of Co was replaced by Ni.

Then, to investigate the cytotoxicity of the SWCNTs produced with the three catalyst mixtures, their effect was evaluated on the behavior of murine MC3T3-E1 preosteoblasts. Either SWCNTs were added on the attached cells or cells were seeded on the SWCNT-covered culture plates. SWCNTs which were added on the attached cells reduced cell viability drastically in a dose-dependent manner. However, the viability of the cells seeded on SWCNTs was only slightly decreased at 24 h, even on those produced with Ni-Co-Y₂O₃. Moreover, cells could proliferate within 48 h. Thus, except mechanical membrane disturbance, thermal plasma grown SWCNTs seemed to induce no severe cytotoxicity on MC3T3-E1 preosteoblasts. Consequently, SWCNTs were purified and their influence on the viability and proliferation of MC3T3-E1 preosteoblasts was determined. The impact of SWCNTs on Smad activation and cell differentiation induced by BMP-2 and BMP-9 was also studied. SWCNTs pre-treatment accelerated the Smad1/5/8 activation induced by both BMP-2 and BMP-9. It did not reduce the viability of preosteoblasts but slightly affected their proliferation at 48 h. Furthermore, after 72 h incubation with BMP-2 or BMP-9, preosteoblasts pre-treated with SWCNTs for 24 h could express genes encoding osteogenic markers such as osterix and osteocalcin and showed high alkaline phosphatase activity. Interestingly, BMP-9 favored the differentiation of preosteoblasts pre-treated with SWCNTs more remarkably than BMP-2. Therefore, combination of BMP-9 with SWCNTs seems to be a promising avenue for bone regeneration.

Keywords: Carbon nanotubes, metallic nanoparticles, induction thermal plasma, cytotoxicity, cell proliferation, mitochondrial enzymatic activity, lactate dehydrogenase, osteogenesis.
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<th>Description</th>
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<tbody>
<tr>
<td>A549</td>
<td>Human alveolar carcinoma epithelial cell line</td>
</tr>
<tr>
<td>ActR</td>
<td>Activin receptor</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>A-GL</td>
<td>Asymmetric Gaussian-Lorentzian function</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Balb/3T3</td>
<td>Mouse fibroblast cell line</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer-Elmer-Teller method</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix loop helix transcription factor</td>
</tr>
<tr>
<td>BMPφ</td>
<td>Bone marrow cell from male ddY mice</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPR</td>
<td>Bone morphogenetic protein receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BSP</td>
<td>Bone sialoprotein</td>
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<td>C2C12</td>
<td>Mouse myoblastic cell line</td>
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<td>C33A</td>
<td>Cervical carcinoma epithelial cell</td>
</tr>
<tr>
<td>C3H10T1/2</td>
<td>Murine mesenchymal multipotent cell</td>
</tr>
<tr>
<td>CB</td>
<td>Carbon black</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFE</td>
<td>Colony-forming efficiency</td>
</tr>
<tr>
<td>cJun</td>
<td>Jun proto-oncogene</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Common-partner Smad</td>
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<td>CT-1</td>
<td>Cardiotropin-1</td>
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<tr>
<td>CTL</td>
<td>Untreated control cells</td>
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<tr>
<td>CVD</td>
<td>Chemical vapor deposition</td>
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<tr>
<td>DAPI</td>
<td>4-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>D-band</td>
<td>Disordered induced band</td>
</tr>
<tr>
<td>Dlx5</td>
<td>Distal-less homeobox 5</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Dimethylformamide</td>
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<td>DWCNT</td>
<td>Double walled carbon nanotube</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDS</td>
<td>Energy dispersive X-ray spectrometer</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>G-band</td>
<td>Tangential mode band</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>H460</td>
<td>Human lung epithelial cell</td>
</tr>
<tr>
<td>HAP</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>hASC</td>
<td>Human adipose stem cell</td>
</tr>
<tr>
<td>HDMEC</td>
<td>Human dermal microvascular endothelial cell</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney epithelial cell</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblast</td>
</tr>
<tr>
<td>HiPco</td>
<td>High pressure carbon monoxide method</td>
</tr>
<tr>
<td>hFOB 1.19</td>
<td>Human fetal osteoblast</td>
</tr>
<tr>
<td>HRSEM</td>
<td>High resolution scanning electron microscopy</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HT22</td>
<td>Hippocampal nerve cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectroscopy</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of DNA-binding/differentiation</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>I-Smad</td>
<td>Inhibitory Smad</td>
</tr>
<tr>
<td>ITP</td>
<td>Induction thermal plasma</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>L-929</td>
<td>Mouse fibroblast cell line</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LTE</td>
<td>Local thermodynamic equilibrium</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MC3T3-E1</td>
<td>Murine preosteoblast</td>
</tr>
<tr>
<td>MCE</td>
<td>Multicellulose ester membrane</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MG-63</td>
<td>Murine osteosarcoma cell line</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRC5</td>
<td>Human fibroblast cell line</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylphenazol-2-y1)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>NR</td>
<td>Neutral red</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>NSERC</td>
<td>Natural Sciences and Engineering Research Council of Canada</td>
</tr>
<tr>
<td>OC</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>Osx</td>
<td>Osterix</td>
</tr>
<tr>
<td>p38</td>
<td>p38 MAPK</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCS</td>
<td>Polycarboxilane</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PF-DAC</td>
<td>Propylene fumarate diacrylate</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethylmethacrylate</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PPF</td>
<td>Poly(propylene fumarate)</td>
</tr>
<tr>
<td>pSmad</td>
<td>Phosphorylated Smad</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa beta</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa beta ligand</td>
</tr>
<tr>
<td>RASMC</td>
<td>Rat aortic smooth muscle cell</td>
</tr>
<tr>
<td>RAW164</td>
<td>Mouse macrophage cell line</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>Murine macrophage-like cell line</td>
</tr>
<tr>
<td>RBM</td>
<td>Radial breathing mode</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RFITP</td>
<td>Radio frequency induction thermal plasma</td>
</tr>
<tr>
<td>rhBMP</td>
<td>Recombinant human BMP</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp tripeptide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROB</td>
<td>Rat primary osteoblast</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ROS17/2.8</td>
<td>Rat osteosarcoma cell line</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor-regulated Smad protein</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Human osteosarcoma cell line</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SLS</td>
<td>Solid-liquid-solid model</td>
</tr>
<tr>
<td>Smad</td>
<td>Small mothers against decapentaplegic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>Smurf</td>
<td>Smad ubiquitin regulatory factor</td>
</tr>
<tr>
<td>SSA</td>
<td>Specific surface area</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>Swiss 3T3</td>
<td>Murine fibroblast cell line</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β activated kinase 1</td>
</tr>
<tr>
<td>TAB1</td>
<td>TAK binding protein 1</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TG</td>
<td>Thermogravimetry</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UV-Vis-NIR</td>
<td>Ultra Violet-Visible-Near Infra Red</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLS</td>
<td>Vapor-liquid-solid model</td>
</tr>
<tr>
<td>WST-1</td>
<td>Mitochondrial enzymatic assay</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-chromosome-linked inhibitor of apoptosis</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
CHAPTER 1 Introduction

1.1 Problematic and project outline

Carbon nanotubes (CNTs) are categorized as nanomaterials. In general, materials with at least one dimension smaller than 100 nm are called nanomaterials. The field that involves using nanomaterials such as CNTs for making various structures of matter with improved and novel properties is called nanotechnology [Poole and Owens, 2003]. Nanomaterials including CNTs have exceptional properties due to their very small size that results in the increase of surface area, placement of the majority of the atoms on the surface, and therefore high reactivity [Jefferson, 2000]. Unique properties of CNTs have made them attractive for many different fields of science and application including tissue engineering [Harrison and Atala, 2007]. For example, CNTs are very promising to be used in the design of biomaterials in contact with bone [Saito et al., 2008].

For CNTs utilization in such applications, they have to be produced with high quality at large-scales. However, Many of the synthesis methods that are used for production of CNTs with high structural quality, such as laser ablation technique, provide only small yields and are either impossible or very expensive to scale up for mass production [Eder, 2010]. Therefore, there is a need for a process that can produce high structural quality CNTs at large-scales.

In 2007, Professor Gervais Soucy’s research group at Université de Sherbrooke reported on large-scale production of high quality single-walled carbon nanotubes (SWCNTs) by radio frequency (RF) induction thermal plasma process [Kim et al., 2007a]. This process takes advantage of the properties of thermal plasma (i.e. high temperature (10 000-15 000 K), large volume of the plasma, high energy density, and presence of reactive species during the operation) to generate CNTs through evaporation of a mixture of carbon black (CB) and metallic particles [Kim et al., 2007a]. This process allows for large-scale production of CNTs because it is possible to treat large amounts of feedstock material continuously and fast due to
the aforementioned characteristics of the thermal plasma [Kim et al., 2007a]. Detailed information about this process can be found in [Kim et al., 2009b].

One important issue regarding utilization of CNTs in different applications is the concerns about the potential toxicity of these nanostructures of carbon due to their very small size, large aspect ratio, and biopersistent chemistry [Aschberger et al., 2010; Donaldson et al., 2006]. CNTs even have been compared to asbestos fibers because of their morphological similarities [Ali-Boucetta et al., 2013; Donaldson et al., 2010a; Kim et al., 2012; Poland et al., 2008].

A huge number of studies have been performed on the toxicity of CNTs (for review see [Aschberger et al., 2010; Firme III and Bandaru, 2010; Hussain et al., 2009; Lam et al., 2006; Shvedova et al., 2009; Smart et al., 2006]). However, although research has gone a long way in this field, the toxicological impacts of CNTs are still under debate mainly because of the heterogeneity of the tested CNTs. Toxicological effects of CNTs have been linked to their properties such as nanometric size and large surface area [Donaldson et al., 2006; Muller et al., 2006; Oberdorster et al., 2004], length and morphology [Donaldson et al., 2006; Muller et al., 2005; Shvedova et al., 2005], hydrophobicity and aggregation state [Soto et al., 2007; Wick et al., 2007] and impurity content and composition [Kagan et al., 2004; Shvedova et al., 2003]. The properties and thus the toxicity profile of CNTs produced by various synthesis methods, carbon sources, and catalysts differ distinctly [Hsieh et al. 2012].

Therefore, the first step towards utilization of a certain type of CNTs in different fields of application is to investigate their toxicity and ensure their safety for human health and environment.

One important parameter in CNT toxicity studies, which is often neglected, is the toxicity of the raw materials (i.e. the carbon source and catalysts) used as feedstock in CNT synthesis processes. For example, depending on the mode of synthesis, metallic catalysts are used for CNT synthesis which will remain in the product as impurities. It has been found that the metallic impurity content of CNTs that varies among samples prepared by different methods (e.g. 0.23 to 26 wt % of Fe in respectively purified and non-purified SWCNTs produced by HiPco (i.e. high pressure carbon monoxide) process) plays an important role in their toxicity.
[Donaldson et al., 2006; Kagan et al., 2004; Kagan et al., 2006; Muller et al., 2006; Shvedova et al., 2003; Tian et al., 2006; Warheit, 2006]. Therefore, investigating the toxic effects of materials used for CNTs production is important not only because their residuals will exist in the final product as a fraction of impurities [Kagan et al., 2006; Tessier and Pascal, 2006; Worle-Knirsch et al., 2006] but also because there is a considerable potential for their exposure in workplaces [Han et al., 2008; Kuhlbusch et al., 2011; Methner et al., 2007]. In fact, the people currently most affected by nanomaterials are those working in the synthesis laboratories [Fiorito et al., 2006a; Pulskamp et al., 2007] who are in direct contact with feedstock materials.

Another important issue in nanotoxicological assessments, which is not considered by many studies, is the comprehensive and detailed characterization of the nanomaterials [Heaweon and Grassian, 2010; Sayes and Warheit, 2009]. Many of the fundamental physical and chemical properties of a given material changes as the particle size decreases to nano-scale ranges, most of the time resulting in completely new and different properties [Warheit, 2008]. Without complete material characterization, it is not possible to correctly attribute the toxic effects to a certain property of the nanomaterial or even the nanomaterial itself because the observed toxicity could be resulted from other things as stated above like impurities [Elsaesser and Howard, 2012].

Once the safety of CNTs is ensured, they can be considered very promising to be used in the design of biomaterials for applications in bone repair and regeneration because of their exceptional mechanical properties, ultralight weight, high stability, and also because they are mainly composed of carbon element which is the main component of the living organisms [Aoki et al., 2007; Saito et al., 2008; Usui et al., 2008].

Indeed, there are about 2.2 million cases of bone surgery per year worldwide that require bone grafting [Giannoudis et al., 2005; Saiz et al., 2013] because of serious bone injuries and musculoskeletal disorders which are increasing due to the aging population as well as the changing human life styles such as physical inactivity, stress, and smoking [World Health Organization, 2003]. Autografts and allografts (i.e. bone taken from another part of one's own body, and bone taken from a donor, respectively) have been used for this purpose as bone
replacements for many years. However, several possible problems including the limited available amount of bone grafts (e.g. autograft) and immunologic rejection of allograft in the host body have necessitated the use of synthetic implants to replace damaged bone [Giannoudis et al., 2005]. Different materials such as metals (e.g. stainless steel and titanium alloys), bioactive (e.g. hydroxyapatite (HAP) and tricalcium phosphate) and nonbioactive (e.g. alumina and zirconia ceramics) ceramics, and natural (e.g. collagen and chitosan) or synthetic (e.g. polycaprolactone (PCL) and polymethylmethacrylate (PMMA)) polymers have been used as bone implants [Saito et al., 2009]. However, several limitations of these biomaterials including lack of integration with the bone tissue (e.g. in metals) as well as insufficient mechanical properties (e.g. in polymers) have resulted in substantial efforts to design new materials for bone replacements. The new generation of biomaterials are called biomimetic materials which can emulate the properties of native bone extracellular matrix (ECM) and control cell adhesion, proliferation, and differentiation [Hench and Polak, 2002].

One of the strategies for developing biomimetic materials is to take advantage of nanotechnology in the design of biomaterials [Rahmany and Van Dyke, 2013]. Benefits of nanotechnology in this regard originate from the fact that the nanometric size of nanomaterials in general resembles that of many components of the bone ECM like collagen type I and HAP crystals [Saiz et al., 2013]. Moreover, compartments of the bone cells involved in their attachment to biomaterials such as focal adhesion complexes range in size from a few to a hundred nanometers [Rahmany and Van Dyke, 2013]. Another example are integrins which are transmembrane receptors in cells with major roles in cell attachment that have a size range from 9 to 12 nm [Xiong et al., 2001; Xiong et al., 2002]. Particularly the exceptional characteristics of SWCNTs make them comparable to many components of the cells. For example, their high aspect ratio, high elastic modulus, and resilience make SWCNTs very similar to the microtubules present in the cytoskeleton of the cells [Pampaloni and Florin, 2008]. Also interactions between SWCNTs result in the formation of bundles with diameters and lengths comparable to the collagen fibers [Zhao et al., 2005]. Interestingly, it has been shown that SWCNTs can be biodegraded in vitro by hypochlorite and reactive radical intermediates of the human neutrophil enzyme myeloperoxidase making them even more
promising for biomedical applications when used at appropriate and degradable concentrations [Kagan et al., 2010].

In this connection, CNTs have been widely investigated for their applications in bone tissue engineering. Many researchers have reported improved adhesion and proliferation of osteoblasts (i.e. bone forming cells) on the substrates made of or containing CNTs [Akasaka et al., 2009; Akasaka et al., 2010; Elias et al., 2002; Giannona et al., 2007]. CNTs have also been used to improve the cytocompatibility of the most widely used implant biomaterials such as titanium and enhance osteoblast growth on them [Bacakova et al., 2007; Sirivisoot et al., 2007]. CNTs can also be applied in the design of biomaterials used as bone substitutes such as natural or synthetic polymeric scaffolds to improve their mechanical properties [Moon et al., 2005; Shi et al., 2005].

1.2 Project definition and objectives

As stated above, before using CNTs in different applications, particularly those directly related to human health, it is necessary to deeply investigate their toxicity. We hypothesized that high quality non-cytotoxic SWCNTs could be synthesized by RF induction thermal plasma using non-cytotoxic feedstock materials and these SWCNTs have the potential to be used in bone applications.

Regarding this hypothesis, the main objective of this study was to minimize the health risks related to the SWCNT production by RF induction thermal plasma through studying the cytotoxicity of the materials present throughout the process (i.e. feedstock materials and SWCNT product) after their complete characterization to evaluate the possibility of using these SWCNTs for orthopedic applications. In order to achieve this objective, four specific objectives were determined in this study.

1.2.1 To evaluate the cytotoxicity of the raw materials used for SWCNT production

SWCNT synthesis by RF induction thermal plasma process requires a carbon source and metallic catalyst particles. Therefore, a feedstock material composed of CB and a ternary
mixture of cobalt (Co), nickel (Ni), and yttrium oxide ($Y_2O_3$) has been used in this process [Kim et al., 2007a].

In order to minimize the health risks from the beginning of the synthesis process, the cytotoxicity of the carbon source and catalysts was investigated after their complete physico-chemical characterization to identify and compare the cytotoxicity related to each material for developing proper sampling and handling methods or eliminating the most harmful ones.

One important point in this regard is that the process used for CNT production could change the physico-chemical properties of the materials and therefore could affect their cytotoxicity. For example, thermal plasma processes can influence the morphology and size of the particles due to the very high temperature profile, rapid cooling, and suppressed grain growth in such systems [Boulos, 2004; Shigeta and Murphy, 2011].

Accordingly, in this project, the effect of the synthesis process was also evaluated on CB and catalyst particles through characterizing and comparing the physico-chemical properties and cytotoxicity of the commercial and thermal plasma treated nanoparticles.

The viability, proliferation, cytoskeleton organization, and apoptosis were evaluated in murine Swiss 3T3 fibroblasts after their exposure to the particles for 24 and 48 h. Material concentrations were graduated to investigate cell toxicity responses. Swiss 3T3 fibroblasts were chosen for this step because they are usually applied for standardized toxicological assessment as model cells which are found in connective tissue throughout the body [Sohaebuddin et al., 2010].

### 1.2.2 To synthesize of SWCNT using different catalysts

Once the cytotoxicity assessment of the feedstock material used for SWCNT synthesis was fully performed, the minimization of the feedstock cytotoxicity and evaluation of the RF induction thermal plasma process capacity to produce high quality SWCNTs using alternative catalysts with minimal cytotoxicity became necessary.

Catalysts play an important role in SWCNTs synthesis [Celnik et al., 2008; Gorbunov et al., 2002; Guo et al., 1995]. To date, a wide range of catalysts have been tested and studied for CNT production using various methods and their roles and effects on the growth of CNTs
have been investigated [Moisala et al., 2003]. Since RF induction thermal plasma is relatively a newly developed process for SWCNT production, few number of catalysts have been tested in this system up to now [Kim et al., 2007a]. It has been reported that any changes in the catalysts nature can directly alter the SWCNT quality [Moisala et al., 2003]. Therefore, a comprehensive study is needed following any alteration in the type or amount of the catalysts to understand the subsequent impacts on the product and be able to optimize the process regarding the new conditions.

In this connection, a series of synthesis experiments were performed to closely examine the influence of different metallic catalysts including Co, Ni, Y$_2$O$_3$ and molybdenum (Mo) at different ratios on the SWCNT final product and evaluate the possibility of applying non-toxic catalyst particles for SWCNT production. Moreover, thermodynamic calculations were carried out using FACTSAGE code (v.6.2) to explore more deeply the influence of type and amount of catalysts on the SWCNTs synthesis by providing further insights into the reaction system in gas phase and liquid solution phase.

1.2.3 To evaluate the cytotoxicity of the produced SWCNTs

Following the evaluation of the raw materials cytotoxicity and the ability of RF induction thermal plasma process to produce SWCNTs using alternative less cytotoxic feedstock materials, the cytotoxicity of the SWCNT final product was evaluated. In fact, the cytotoxicity of RF induction thermal plasma grown SWCNTs has never been studied before.

As previously mentioned, CNTs synthesized by various methods are expected to have different properties. Indeed, the properties of CNTs are very dependent not only on the synthesis method applied [Hussain et al., 2009] but also on the starting materials used for their production (i.e. carbon sources [Alinejad et al., 2010; Chiang et al., 2001], and metallic catalysts [Ajayan et al., 1993; Dai et al., 1996; Journet et al., 1997; Kumar and Ando, 2010a; Lambert et al., 1994]). In addition to CNTs, the as-produced product usually contains impurities [Wick et al., 2007]. The amount and chemistry of these impurities are also dependent on the synthesis process [Journet et al., 2012]. Therefore, It is impossible to attribute the cytotoxicity results obtained for a certain CNTs material produced by a specific
system (i.e. process and feedstock material) to another one because of their different characteristics and therefore distinct toxicity profiles [Hsieh et al., 2012].

RF induction thermal plasma synthesis method is a very promising process for continuous large-scale production of high quality SWCNTs. SWCNTs produced by this process are already commercialized by Raymor industries Inc. (Canada) and are expected to be used widely in various applications.

In the present study, the cytotoxicity of SWCNTs synthesized by RF induction thermal plasma was evaluated for the first time. Moreover, by applying three catalyst mixtures (Ni-Co-Y$_2$O$_3$; Ni-Y$_2$O$_3$; Ni-Mo-Y$_2$O$_3$) for SWCNT synthesis, the effects of different catalysts were investigated on the properties and cytotoxicity of the SWCNTs material.

Since applications related to bone tissue is the interest of this study, MC3T3-E1 murine preosteoblasts were chosen as model cells. The typical osteoblastic differentiation sequence of these cells is similar to that of human cells [Bergeron et al., 2009] and this cell line is often used for in vitro biomaterial testing [Vandrovcova and Bacakova, 2011]. Besides, since CNTs can be used in bone applications as dispersed in suspension [Mao et al., 2013; Mendes et al., 2010], or embedded in composites [Van Der Zande et al., 2010] and substrates [Li et al., 2012], we investigated the behavior of bone cells in contact with SWCNTs in both suspended or immobilized form.

1.2.4 To evaluate the cytotoxicity of the purified SWCNTs and their effect on bone cells

In order to achieve the highest possible effectiveness of CNTs in many applications, they have to be purified. Therefore, a well-defined purification protocol is necessary for effective removal of the impurities. We have recently developed a purification protocol by which the main impurities of RF induction thermal plasma grown SWCNTs were successfully minimized through a multistep procedure [Kim et al., 2009a].

Osteoinduction (i.e. the ability to recruit stem and osteoprogenitor cells to the site and their stimulation to undergo osteogenic differentiation [Albrektsson and Johansson, 2001]) is a crucial property of a successful bone related biomaterial. The biomimetic materials developed
for bone replacements can contain growth factors (GFs) to favor bone tissue regeneration [Salgado et al., 2004]. GFs are a group of signaling molecules that bind to specific transmembrane receptors on the cells and initiate an intracellular signaling that affects cell proliferation, and differentiation [Salgado et al., 2004].

Among various GFs, bone morphogenetic proteins (BMPs) which are a sub-family of transforming growth factor beta (TGF-β) superfamily are very important in bone repair due to their exceptional osteoinductivity [Canalis et al., 2003]. BMP-2 which is approved by the United States Food and Drug Administration (FDA) is the most frequently used BMP in clinical bone repair [Burkus et al., 2004; McKay and Sandhu, 2002]. However, BMP-9 has been shown to be a more effective osteogenic molecule than BMP-2 [Kang et al., 2004].

The use of CNTs in bone tissue engineering requires a better understanding of the effect of CNTs on the ability of bone cells to respond to their environment, especially GFs including BMPs. However, only few studies have investigated the effect of CNTs in combination with BMP-2 on bone cells in recent years [Li et al., 2009; Li et al., 2012; Nayak et al., 2010; Van Der Zande et al., 2010]. Furthermore, the effect of CNTs on bone cell responses to BMP-9 has not been evaluated yet.

In this study, we purified RF induction thermal plasma SWCNTs, characterized their morphology and composition and then analysed their effects on the behaviour of MC3T3-E1 preosteoblasts in response to equimolar concentration (1.92 nM) of BMP-2 or BMP-9. This concentration of BMPs was previously shown to induce osteogenic differentiation in MC3T3-E1 preosteoblasts as well as human mesenchymal stem cells (MSCs) effectively [Bergeron et al., 2009; Krattinger et al., 2011]. The lack of cytotoxicity of SWCNTs was first confirmed by following cell viability and cytoskeleton organization up to 72 h. Next, the BMP signalling sequences were evaluated in MC3T3-E1 preosteoblasts pre-treated with or without SWCNTs and stimulated with BMP-2 or BMP-9. The differentiation of preosteoblasts was verified by quantifying the expression of genes encoding osteogenic markers and measuring the alkaline phosphatase (ALP) activity.
1.3 Original contributions

As previously stated, prior to the possible use of SWCNTs in different fields of application, their associated health risks have to be completely investigated. In the case of SWCNTs, however, this is a multidisciplinary task that needs a good understanding of biology and material characterization. To fully characterize SWCNTs, the knowledge about their synthesis process and the feedstocks used for their production is necessary. However, although many studies have been performed so far on CNTs cytotoxicity, the conclusions are controversial and somehow misleading. This is because the focus has been mainly on the final CNT product which is often purchased without having enough information about their properties and the raw materials and the process used for their synthesis. The main originality of the present work arises from the fact that synthesis, characterization, cytotoxicity assessment, and evaluating the potential application of the RF induction thermal plasma grown SWCNTs have been studied all together. This provides the opportunity to know the specifications of the materials used and synthesized, understand the observed cellular effects and attribute them to a certain property or component of the tested sample, and also improve the system in order to produce SWCNTs with better qualities in a safe manner. Other main original contributions of this study are specified below.

- The effect of SWCNT synthesis process that is the RF induction thermal plasma was deeply investigated for the first time on the physicochemical and cytotoxic properties of the starting materials (i.e. catalysts and carbon source) used in the SWCNT production.
- Non-toxic Mo catalyst was employed for the first time to synthesize SWCNTs using RF induction thermal plasma method. Moreover, new different catalyst ratios in the feedstock mixture were tested.
- The cytotoxicity of the SWCNT final product synthesized by RF induction thermal plasma system using three different catalysts was assessed.
- The effect of RF induction thermal plasma SWCNTs in combination with BMP-2 on the proliferation and differentiation of the bone cells was investigated.
- The effect of CNTs/BMP-9 combination was investigated for the first time on the proliferation and differentiation of the bone forming cells.
1.4 Plan of the document

This thesis contains seven main chapters. Chapter 1 is a brief introduction to the project describing the context, and objectives.

Chapter 2 is a review of the published work on the CNTs, their synthesis processes, growth mechanisms, properties, and their applications particularly in bone regeneration. Moreover, bone tissue and the important factors in bone regeneration are described briefly.

Chapter 3 presents the cytotoxicity assessment of the commercial materials from which SWCNTs are produced. Moreover the effect of the synthesis process on the physico-chemical properties and cytotoxicity of these materials was investigated. This chapter has been accepted for publication in Nanotoxicology.

Chapter 4 describes systematic thermodynamic and experimental studies on the effects of the catalysts type and content on SWCNTs synthesis by RF induction thermal plasma process. The feasibility of this process to produce SWCNTs with different catalyst mixtures was evaluated, the role of each catalyst in SWCNTs synthesis process was studied, and solutions for effective use of non-toxic catalysts in synthesizing high quality SWCNTs were proposed. This chapter is published in journal of physics: conference series.

Chapter 5 presents evaluation of the effects of RF induction thermal plasma grown SWCNTs on the attachment, viability, and proliferation of murine preosteoblasts. The influences of three catalyst mixtures on the properties and cytotoxicity of SWCNTs as well as the effect of cell-SWCNTs exposure methods on the results of cytotoxicity assessments were also evaluated. This chapter is accepted for publication in journal of applied toxicology.

In chapter 6 the effect of the purified RF induction thermal plasma grown SWCNTs on the viability, proliferation, and differentiation of murine preosteoblasts in presence of BMP-2 and BMP-9 is presented and RF induction thermal plasma grown SWCNTs as highly potent and promising materials for bone related applications. This chapter is accepted for publication in journal of biomedical nanotechnology.

Chapter 7 summarizes the main results and the proposed future works.
CHAPTER 2 Literature review

Before explaining the methodology and the achieved results (published or accepted) of this study, the description of CNTs in terms of structure, synthesis, properties, and their applications particularly in orthopedics is required. To better understand the effect of CNTs on the bone forming cells and why they may be used in bone applications with or without growth factors, the histology of the bone tissue, its cells, and the factors involved during its repair will be briefly described.

2.1 Carbon allotropes

Carbon (C) is one of the most abundant elements in the world and is present in the structure of all living organisms. At atomic level, carbon has six electrons among which two reside at N=1 energy state in Is orbital and do not participate in chemical bonding. The four remaining electrons which form the chemical bonds of carbon with other atoms, distribute at N=2 energy state, one in 2s orbital and the last three in 2p orbitals. Carbon atom can make different allotropes because it is capable of bonding with other atoms through different hybridization levels which are (a) sp (linear), (b) sp² (planar), and sp³ (tetrahedral) [Poole and Owens, 2003]. Solid phase carbon has been known in the form of amorphous carbon, graphite, and diamond for many years (Figure 2.1). In 1985, Smalley and coworkers discovered a new allotrope of carbon, C₆₀ fullerene or buckyball, which is a molecule composed of sixty carbon atoms bonded together in the form of 12 pentagons and 20 hexagons [Kroto et al., 1985]. In 1991, Iijima observed a new arrangement of carbon atoms in the form of rolled single atomic layers of graphite (i.e. graphene sheet) in the soot synthesized by an arc discharge set-up basically used for fullerene synthesis [Iijima, 1991]. These new crystalline carbon structures were named CNTs due to their cylindrical morphology and their diameter that lied in the range of nanometers. Moreover, since these nanotubes were composed of several graphitic layers, they were named multi-walled carbon nanotubes (MWCNTs). In 1993, another type of CNTs was discovered by addition of transition metal catalysts to the carbon source in the arc discharge set-up [Bethune et al., 1993; Iijima and Ichihashi, 1993]. These new CNTs were composed of only one graphitic layer and thus were named SWCNTs. Different atomic
organizations in the structure of carbon allotropes give them distinct properties. For example, in diamond, carbon atoms make σ bonds with four other carbon atoms through sp\(^3\) hybridization. In graphite, fullerene, and CNTs, three σ bonds and one π bond is formed between one carbon atom and three others. The π bonds are located perpendicular to the plane of the graphene sheet and their electrons are not restricted and can move freely along the axis of the CNT [Dervishi et al., 2009]. That is the reason why diamond is an electric isolator while graphite, fullerene, and CNTs are electric conductors [Dervishi et al., 2009].

![Diagram of carbon allotropes](image)

Figure 2.1 Carbon forms different solid allotropes which are amorphous carbon, diamond, graphite, fullerene, and CNTs. Figure created using [Nanotube Modeller\(^3\), JCrystalSoft, version 1.7.3].

### 2.2 Carbon nanotubes (CNTs)

CNTs are cylindrical macromolecules of carbon. Their walls are composed of hexagonal lattices made up of sp\(^2\)-bonded carbon atoms and the closed-cap ones have a half fullerene molecule as their cap [Balasubramanian and Burghard, 2005]. CNTs are either multi- or single-walled depending on the number of cylinders they comprise. MWCNTs are made up of several concentric cylindrical single atomic layers of crystalline graphite, also known as graphene sheet, while SWCNTs are composed of only one layer of graphene sheet [Ajayan
The spacing distance between the walls of a MWCNT is 0.34 nm [Ajayan and Ebbesen, 1997]. Diameter of MWCNTs can reach 100 nm whereas that of SWCNTs lies between 0.7 to 3 nm while both MWCNTs and SWCNTs lengths can reach to several microns [Dervishi et al., 2009] or even millimeters [Kishimoto et al., 2005]. The atomic structure of a CNT is defined by a circumferential vector (i.e. $\vec{C}_h = n\vec{a}_1 + m\vec{a}_2$) which is perpendicular to the CNT axis and is specified by a pair of indices (n, m) denoting the number of unit vectors, $\vec{a}_1$ and $\vec{a}_2$, in the hexagonal honeycomb lattice of the graphene sheet (Figure 2.2) [Thostenson et al., 2001]. The CNT diameter, $d_t$, can be measured in terms of the (n, m) integers as $d_t = \frac{|\vec{C}_h|}{\pi} = \frac{\sqrt{3}}{\pi} a_{cc}(m^2 + mn + n^2)^{0.5}$ where $a_{cc}$ is the nearest-neighbor C–C distance (1.421 Å in graphite), and $|\vec{C}_h|$ is the length of the circumferential vector [Terrones, 2003].

Another important feature in the CNT structure is the chiral angle ($\theta$) which is the angle between the circumferential vector $\vec{C}_h$ and $\vec{a}_1$ and can be between 0° and 30°. According to the chiral angle, CNTs are categorized into armchair, zigzag, and chiral (Figure 2.2) [Thostenson et al., 2001]. The C–C bonds on opposite sides of each hexagon are perpendicular to the tube axis in armchair CNTs, while in the zigzag ones these bonds are parallel to the tube axis. In chiral CNTs, the C–C bonds make an angle ($\theta$) with the tube axis [Terrones, 2003].
2.3 Synthesis of CNTs

A number of processes have been developed for CNT synthesis among which arc discharge, laser ablation, chemical vapor deposition (CVD), and RF induction thermal plasma are the most well-established and promising ones (for review see [Baddour and Briens, 2005; Joumet et al., 2012]). By introducing suitable catalysts, each of these methods can be used for SWCNT production.

2.3.1 Arc discharge

The arc discharge method is one of the most common methods for CNTs production (for review see [Ando and Zhao, 2006]). Indeed, Iijima’s report on the synthesis of MWCNTs by arc discharge in 1991 introduced this new class of solid carbon widely to the world [Iijima, 1991]. The first reported SWCNTs were synthesized by arc discharge in 1993 [Bethune et al., 1993; Iijima and Ichihashi, 1993]. This method comprises two graphitic electrodes placed at a distance of few millimeters from each other in an inert gas (e.g. helium (He)) environment (Figure 2.3 a). By application of a suitable voltage (e.g. 20 V) and current density (e.g. 150 A/cm²), the plasma arc discharge vaporizes the anode. For SWCNT production, presence of metallic catalysts is in fact necessary. Metals such as Co, Ni, and iron (Fe), or their combinations have been used for SWCNT production [Ando and Zhao, 2006]. The anode will be consumed and the well-crystallized CNTs will form on the cathode along with impurities like amorphous carbon which will be present in the final product [Joumet et al., 1997]. One major drawback of this method is the consumption of the carbon source that limits its use for continuous production at large scales.

2.3.2 Chemical vapor deposition (CVD)

CVD method which involves catalyst assisted thermal decomposition of hydrocarbons has become one of the most popular methods for CNT synthesis due to its relatively easy process and possibility of large scale production (for review see [Kumar and Ando, 2010b]). In 1996, isolated SWCNTs were grown using disproportionation of carbon monoxide at 1 200 °C, with Mo particles as catalysts by CVD method [Dai et al., 1996]. In this process, a hydrocarbon vapor (CₓHᵧ) enters an oven containing a heat resistant tube and decomposes by passing upon
a substrate (e.g. alumina or silica) on which catalysts are dispersed (Figure 2.3 b). The elements mostly used as catalysts in this process are Fe, Co, and Ni [Kumar and Ando, 2010b]. The catalysts act as nucleation growth sites of the CNTs and also assist the hydrocarbon decomposition [Kumar and Ando, 2010b]. The operating environment is filled with inert gases and CNTs are grown on the catalysts and collected. Low temperature CVD processes (i.e. 600-900 °C) mostly result in MWCNTs while high temperature CVD processes (i.e. 900-1200 °C) mostly result in SWCNTs [Kumar and Ando, 2010a]. The SWCNTs made by this process are usually aligned but they have defective structures [Ren and Huang, 1998; Thostenson et al., 2001].

### 2.3.3 Laser ablation

Laser ablation is another method for CNT production by which the first reported high quality SWCNTs were synthesized [Guo et al., 1995; Thess et al., 1996]. In this method, a graphitic target placed in an oven containing a quartz tube is evaporated by a laser beam (Figure 2.3 c). The system operates at ~ 1200 °C and the environment of the oven is filled with inert gases such as argon (Ar) and He that push the carbon atoms from the high temperature region towards a cooling finger usually made of copper and located at the end of the oven on which CNTs are formed. For SWCNT production, metallic catalysts namely Ni and Co or their combinations should be added to the carbon source [Kingston et al., 2004]. This method is mainly used for production of highly pure and well crystallized defect free CNTs at lab scale [Kingston et al., 2004]. The main obstacle for its scaling up is the high power consumption at large scale and also the need for interruption of the process for recharging the carbon source [Rafique and Iqbal, 2011].
2.3.4 Radio frequency induction thermal plasma process

Definition of the plasma

Plasma consists of a mixture of electrons, ions, and neutral particles in which negative and positive charges balance each other. Therefore, the plasma is overall electrically neutral which is also known as quasi-neutrality [Boulos, 1991]. Plasmas are categorized into thermal and cold categories. In thermal or equilibrium plasmas, the temperature of the heavy particles (i.e. ions and neutrals) and that of the electrons are equal which results in high energy density local thermodynamic equilibrium (LTE) in thermal plasmas [Boulos, 1991]. Plasmas produced by RF inductively coupled discharges are typical examples of thermal plasmas [Boulos, 1991]. Thermal plasmas are often used in materials processing because of their high energy density that enables heating, melting, and, in some cases, vaporizing the material to be treated [Boulos, 2012; Boulos, 1991].

Cold or non-equilibrium plasmas, on the other hand, have lower energy densities compared to thermal plasmas and the temperature of the electrons is much higher than that of heavy particles in these plasmas [Boulos, 1991]. In material processing, low-pressure cold plasmas are used in plasma etching and deposition processes as well as in plasma surface modifications due to the presence of reactive and chemically active species [Boulos, 1991].
Specifications of the RF induction thermal plasma process for CNT synthesis

RF induction thermal plasma process has shown a great promise in mass production of high quality SWCNTs [Kim et al., 2007a]. The Schematic diagram of the set-up originally used for production of SWCNTs by this process is illustrated in Figure 2.4. In this process, the energy needed for vaporization of the carbon source is provided by a RF induction plasma torch (TEKNA PS-50, Tekna Plasma Systems, Inc.) in which the electromagnetic field produced by an induction coil generates thermal plasma from a gas (Figure 2.4 a). The RF induction torches do not contain any electrode which is advantageous because possible contamination from contact with the electrode material will be avoided. Furthermore, a wide range of gases including inert or corrosive ones can be used in the process, and continuous operation will also be possible [Fauchais and Vardelle, 1997]. The RF induction torch is composed of a 3 to 7 turn induction coil which is connected to a power supply and a water-cooled quartz or ceramic confinement tube. Three gas streams are introduced into the torch: (a) sheath gas which protects the confinement tube from the high temperature, (b) central gas which stabilizes the plasma, and (c) powder carrier gas which transports the solid feedstock material continuously introduced into the system through a water-cooled probe (Figure 2.4). Pure Ar or its mixture with other gases such as He is of great interest for the plasma gas mainly because it is ionized easily [Boulos, 1991]. He is used as the sheath gas because of its higher thermal conductivity (1.35 W/mK at 5 000 K) compared to Ar (0.28 W/mK at 5 000 K) which results in more effective heat exchange between the high temperature plasma and the injected feedstock [Boulos et al., 1994]. Therefore, more vaporization of the carbon and metallic particles is obtained in a given time [Kim et al., 2007a].
The system comprises a tubular reactor of two distinguished parts (i.e. reaction and quenching chambers) which is connected to a filtration chamber. The reaction and quenching chambers are double-walled cylinders of 50 cm length and 15 cm diameter. The reaction chamber is surrounded by graphite inserts for higher temperature profile achievement. The filtration chamber contains three metallic filters to separate the solid product from the gaseous ones which will be exhausted through a vacuum pump. Very high temperature and large volume of the induced plasma by the RF induction torch makes this system ideal for CNT synthesis because the particles will have enough residence time passing through the hot regions of the plasma to melt and vaporize and generate precursors for CNT formation [Kim et al., 2007a]. Furthermore, the high temperature environment and the lack of consumable electrodes as the carbon source results in the continuous production of high quality CNTs at large scale [Kim et al., 2009b].
Materials used for SWCNT production by RF induction thermal plasma process

CB has been used for large scale production of SWCNTs by RF induction thermal plasma process because of its industrial availability and low cost as well as its composition which contains mainly carbon element that eliminates production of unwanted by-products [Alinejad et al., 2010]. Co and Ni have been widely used as catalysts for CNT production because at high temperatures carbon is highly soluble and diffusible in these metals [Kumar and Ando, 2010a]. Moreover, stronger adhesion of Co and Ni with the growing CNTs compared to other transition metals favors the formation of high curvature CNTs such as SWCNTs [Ding et al., 2008]. Addition of $\mathrm{Y_2O_3}$ is also favorable since the presence of yttrium (Y) results in the formation of yttrium carbide ($\mathrm{YC_2}$) through an exothermic reaction [Kim et al., 2007a; Meyyappan, 2005]. The formation of $\mathrm{YC_2}$ results in an increase in catalyst particle temperature and consequently induces a longer liquid state of these particles [Kim et al., 2007a]. A longer liquid state for catalysts favors carbon atom dissolution in them and enhances CNTs formation [Kim et al., 2007a]. Furthermore, the released oxygen can burn away the excess amount of amorphous carbon decreasing the proportion of impurities and avoiding encapsulation of the catalyst particles during the growth process [Kim et al., 2007a]. Moreover, it is suggested that due to its low ionization potential Y increases radiative heat transport in plasma which is one of the most important transport phenomena in thermal plasmas [Kim et al., 2009b].

Table 2.1 summarizes the principles, benefits, and weaknesses of different SWCNT synthesis processes.
<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>SWCNT features</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF induction thermal</td>
<td>Carbon atom generation from CB by thermal plasma of He and Ar with T&gt;1 0000 K in an inert environment</td>
<td>Excellent CNTs quality</td>
<td>Moderate purity</td>
<td>d: 1.2-1.6 nm</td>
</tr>
<tr>
<td></td>
<td>- CNTs formation in the presence of Co, Ni, and Y$_2$O$_3$ catalysts</td>
<td>- Easy to scale up</td>
<td>CNTs - Purification required</td>
<td>l: up to ~ 2 μm</td>
</tr>
<tr>
<td></td>
<td>- Collection of CNTs on porous filters</td>
<td>- Continuous high production rate (up to 1 kg/day)</td>
<td>- Possibility of in situ purification and functionalization</td>
<td>- Defect free, entangled, and bundled CNTs</td>
</tr>
<tr>
<td>Laser ablation</td>
<td>Carbon atom generation from evaporation of a graphite target containing catalysts by a pulsed laser beam in an inert environment T&gt;1 200 °C</td>
<td>Excellent CNTs quality and purity</td>
<td>Very expensive process</td>
<td>d: 1.2-1.7 nm</td>
</tr>
<tr>
<td></td>
<td>- Collection of CNTs on a water-cooled collector</td>
<td>- Low production rate (maximum ~ 50 g/day)</td>
<td>- Difficult to scale up</td>
<td>l: up to several hundreds of nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Defect free</td>
<td>- Defect free amorphous carbon free, bundled CNTs</td>
<td></td>
</tr>
<tr>
<td>CVD</td>
<td>Carbon atom generation from decomposition of a hydrocarbon gas in a furnace with T&gt;1 100 °C in an inert environment</td>
<td>Excellent CNTs purity</td>
<td>Poor quality</td>
<td>d: up to 4 nm</td>
</tr>
<tr>
<td></td>
<td>- CNTs formation on a cooler substrate containing catalysts</td>
<td>- Simple process, easy to scale up</td>
<td>defected CNTs</td>
<td>l: up to few mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Continuous high production rate (up to 50 kg/day)</td>
<td></td>
<td>- Open cap</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- individual</td>
</tr>
<tr>
<td>Arc discharge</td>
<td>Carbon atom generation from a positive graphitic electrode containing catalysts by an electric arc discharge with T&gt;3 000 °C</td>
<td>Good CNTs quality and purity</td>
<td>Purification required</td>
<td>d: 1.2-1.5 nm</td>
</tr>
<tr>
<td></td>
<td>- CNTs formation on the negative electrode</td>
<td>- Low production rate (maximum 120 g/day)</td>
<td>- Closed cap</td>
<td>l: up to 20 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Difficult to scale up</td>
<td>relatively defect free, bundled CNTs</td>
<td></td>
</tr>
</tbody>
</table>

2.4 Mechanisms of SWCNTs formation and growth

Since SWCNTs have been synthesized with diverse methods and operating conditions, different models have been proposed for the mechanism of their growth so far among which vapor-liquid-solid (VLS) [Saito et al., 1994], solid-liquid-solid (SLS) [Gorbunov et al., 2002] and the scooter [Thess et al., 1996] models are the most well-known. All of these models consider formation of SWCNTs in the presence of catalysts. Therefore, the role of catalysts is crucial in these models for defining a reliable mechanism for SWCNT formation. VLS model
was firstly proposed by Saito et al. [Saito et al., 1994] from the observations made by arc discharge method and later extended by Gavillet et al. [Gavillet et al., 2002] (Figure 2.5 a). The model consists of two steps: (a) nucleation and (b) growth of nanotubes. Carbon-saturated metal nanoparticles formation is the first step in the nucleation process [Gavillet et al., 2002]. In the case of catalytic methods such as CVD, the metallic nanoparticles are pre-formed, and their super saturation occurs in a multi-step process [Gavillet et al., 2002]. The carbon source is firstly decomposed and the resultant carbonaceous structures, mainly molecular aggregates, adsorb on the surface of the pre-formed metallic nanoparticles and become dissolved in the liquid metal. In high temperature methods, however, prior to the formation of metal liquid phase, the carbonaceous structures are formed from the condensing carbon vapor [Gavillet et al., 2002]. The liquid metallic nanoparticles can then dissolve large amounts of carbon atoms because the solubility of carbon in metal particularly in nano-sized metal liquid is relatively high [Krivoruchko and Zaikovskii, 1998]. During the cooling process, the solubility of carbon in metals decreases which results in segregation of excess carbon from the liquid metal. This step of the mechanism is tightly dependent on metal nanoparticles nature and size, carbon concentration, temperature gradient during the cooling process, and the operating pressure. According to two competing paths, carbon crystallizes either in the form of graphite layer or as a nanotubes (Figure 2.5 a) [Gavillet et al., 2002]. If the segregation velocity is low, the graphite layer (i.e., equilibrium structure of carbon) is formed on the metal nanoparticles. In contrast, a rapid segregation inhibits the formation of graphite layer and favors the nucleation of nanotubes on the surface of metal nanoparticles. Once the nanotube nucleation begins, its growth occurs through integration of carbon at the root which is called the "root growth mechanism" [Gavillet et al., 2002]. The carbon is then continuously supplied from the vapor phase through diffusion process in order for the nanotube growth to continue. Once the solidification occurs at low local temperature, the nanotube growth terminates. If the balance between the SWCNT growth kinetic, cooling kinetic, and carbon supply is perturbed, the carbon vapor can partially condense into amorphous carbon flakes or graphitic sheet consisting of few layers. This condition causes an intermediate situation between nanotube nuclei and long bundles which is referred to as the sea-urchin structure [Gavillet et al., 2002]. In this case nanotube bundles are formed in any direction on the metal particle preventing the
sufficient carbon supply from the gas phase for continuous growth and resulting in short-length nanotube bundles (Figure 2.5 a) [Gavillet et al., 2002].

Unlike the VLS model, in the SLS model proposed by [Gorbunov et al., 2002] (Figure 2.5 b), the existence of carbon vapor is not essential. Instead, the model suggests that condensed amorphous carbon supplies the carbon atoms for the growth of SWCNT. SWCNT growth is basically considered as a condensed state transformation of one solid carbon form to another. Molten catalyst is surrounded by amorphous carbon which supplies the carbon by dissolution process and the excess carbon atoms come to the surface of the particle by precipitation process. During the evolution of these atoms, graphitic structures with defects and dangling bonds are formed on the surface which is not energetically favorable. Therefore, these graphitic sheets in the form of graphene will then become curved, and form the SWCNT nuclei. As the participation continues, these nuclei are transformed to tubular structures in the form of nanotubes.

![Figure 2.5](image_url) Proposed SWCNT growth mechanisms: (a) VLS, (b) SLS, and (c) scooter mechanisms. Adapted from [Gavillet et al., 2002; Gorbunov et al., 2002; Krueger, 2010].

According to the observations from laser-ablation method, the "Scooter" mechanism was proposed by [Thess et al., 1996] to explain the high yield and uniformity of synthesized SWCNTs. As schematically shown in Figure 2.5 c, a single catalyst atom will be absorbed to the open edge of curving single-layered graphite with less than 50 atoms. At sufficiently high temperatures, catalysts exist in the form of single atoms or very small clusters which are situated at the open end of a growing nanotube. The growth continues by insertion of small
carbon clusters such as \( \text{C}_2 \) from gas phase through the metal atom. The position of metal atom is not static and it moves back and forth like it scoots along the circumference of the growing nanotube [Krueger, 2010]. This scooting process helps annealing of any carbon structures that are not energetically favorable and keeps the tube open. As the process continues, further metal atoms concentrate on nanotubes end and finally aggregate to small clusters. Consequently, at certain size the bonding energy between metal and carbon atoms decreases so that the metal particle separates from the nanotube tip. Accordingly, the tube's end becomes closed and the growth will be terminated [Krueger, 2010].

Among the described mechanisms, the VLS model is the best suited for explaining the growth of SWCNTs in RF induction thermal plasma process [Kim et al., 2009b].

2.5 Properties of CNTs

When at least one dimension of a material is reduced to nanometric ranges (i.e., <100 nm), dramatic changes occur in its properties [Poole and Owens, 2003]. In the case of CNTs, two dimensions out of three are below 100 nm so that they can be considered as molecular quantum wires or one-dimensional systems [Dekker, 1999]. Unique thermal and electronic properties of CNTs originate from their physics at atomic level while their exceptional mechanical properties are majorly related to the very strong chemical bonds among carbon atoms (i.e., C–C \( \text{sp}^2 \)). In this section, properties of CNTs will be discussed in more details.

2.5.1 Electronic properties

CNTs can be metallic or semiconductive depending on their chirality indices \((n, m)\) [Saito et al., 1992a; Saito et al., 1992b]. Specifically, the SWCNTs for which \(|n - m|\) is equal to \(3q\) are metallic, and those for which \(|n - m|\) is equal to \(3q \pm 1\) are semiconducting, where \(q\) is an integer [Dresselhaus et al., 2002]. Hence, this is the chirality of CNTs which determines their electronic properties [Saito et al., 1992a; Saito et al., 1992b]. In a SWCNT sample, one third of the tubes are usually metallic and the rest are semiconductive [Poole and Owens, 2003]. Exceptional electronic properties of CNTs originate from the quantum mechanical nature of these materials in the radial direction which falls into nanometric range close to the spacing distance between the atoms. Their axial direction reaches several micrometers while their
radial direction is in nanometer range. Therefore, the delocalized electrons move freely along
the CNT axis but they are very confined in the radial direction [Poole and Owens, 2003].
CNTs can carry very high currents with almost no heating because electronic transport in
metallic SWCNTs and MWCNTs occurs without scattering, known as ballistic transport,
along the axis [Baughman et al., 2002]. The current density in an individual SWCNT exceeds
10⁹ A/cm² which is 1000 times more than that in copper [Eder, 2010]. A more complete
review on the electronic properties of CNTs is given by [Dresselhaus et al., 2004].

2.5.2 Thermal properties
Due to their one dimensional nanometric and graphitic structure, CNTs possess extraordinary
thermal properties [Balandin, 2011]. Thermal transport in CNTs is exceptionally effective due
to quantum confinement in their one dimensional structure [Ciraci et al., 2001]. Moreover, the
C-C bond in the structure of a CNT exhibits a very high frequency vibration which allows for
easy propagation of phonons (i.e. quasi particles or the quantized vibrational energy in a
crystalline network) along the CNT axis and consequently enhanced thermal conductivity
[Balandin, 2011]. The measured thermal conductivity for an individual SWCNT and MWCNT
are ~7000 and 3000 W/mK at room temperature (RT) respectively which are noticeably
greater than that of natural diamond and the basal plane of graphite with 2000-2500 W/mK
thermal conductivity at RT [Baughman et al., 2002].

2.5.3 Chemical properties
Chemical reactivity of a CNT is higher than that of graphite [Eder, 2010]. A complete CNT is
considered to comprise a half fullerene residing at its tip and a rolled-graphene tubule as the
side walls. The characterization of the CNT chemistry therefore implies to take into account
the chemistry of both fullerene and curved graphene sheet. The curvature of CNTs is also a
key parameter in the chemistry of these materials since it induces local strain due to the
structural folding resulting from pyramidalization (fulleronic part) and π orbital misalignment
(side walls) in the CNT network [Banerjee et al., 2005; Niyogi et al., 2002]. CNTs with
smaller diameters are expected to be more reactive due to their higher curvature [Banerjee et
al., 2005]. Reactivity of CNTs also depends on their surface area. SWCNTs possess a very
high surface area (i.e. up to few hundred \( \text{m}^2/\text{g} \) for as-produced and to up to 1 000 \( \text{m}^2/\text{g} \) for purified SWCNTs) [Lafi et al., 2005]. The calculated specific surface area (SSA) for as-produced and purified SWCNTs synthesized by RF induction thermal plasma process have been measured to be 122 and 480 \( \text{m}^2/\text{g} \) respectively [Kim et al., 2009a]. The surface area of a SWCNT sample depends on the diameter, length, being open or closed cap, and also being individual or in a bundle [Agnihotri et al., 2005]. For example, the only available area for an individual CNT with closed cap is its surface whereas molecules or atoms can also react with the internal surface of a CNT with opened cap [Hu et al., 2003].

2.5.4 Mechanical properties

The C-C covalent bond in graphite is one the strongest bonds in nature [Ajayan and Ebbesen, 1997]. Therefore, a very high strength has been expected for CNTs. Indeed, Young’s modulus, which characterizes the elastic flexibility of a material [Poole and Owens, 2003], has been measured to be the highest value for CNTs compared to any other material [Poole and Owens, 2003]. The experimental measurements performed on MWCNTs and SWCNTs indicated average Young’s moduli of 1.8 and 1.25 TPa respectively [Salvetat et al., 1999b; Treacy et al., 1996; Wong et al., 1997]. Moreover, diameter of CNTs influences their Young’s modulus (e.g. 1.4 and 0.7 TPa for SWCNTs with respectively 1 and 2 nm diameter) [Krueger, 2010]. Mechanical strength of CNTs is 20 times higher than that of steel having an elasticity Young’s modulus of 0.21 TPa [Poole and Owens, 2003]. Tensile strength, which determines the amount of stress needed to pull a material apart, has been measured to be 45 TPa for CNTs [Poole and Owens, 2003]. For high-strength steel alloys this value is about 2 TPa [Poole and Owens, 2003].

The mechanical properties of a CNT also strongly depend on its atomic structure. The closer the structure of a CNT is to the ideal graphene sheet with hexagonal carbon lattices, the higher the mechanical properties will be [Coleman et al., 2006]. Therefore, the CNTs synthesized by laser ablation, RF induction thermal plasma, and arc discharge will have the highest mechanical properties respectively due to the ability of these techniques to produce high structural quality CNTs [Salvetat et al., 1999a]. However, CVD grown CNTs are expected to have significantly reduced values compared to those mentioned previously [Salvetat et al.,
1999a]. For example, high values of ~ 1 TPa and ~ 50 GPa has been reported for the elastic modulus and tensile strength of arc discharge CNTs respectively [Coleman et al., 2006], while the values of Young's modulus lie between 50 GPa and 0.45 TPa for CVD grown MWCNTs [Salvetat et al., 1999c; Xie et al., 2000] and their tensile strength is about 4 GPa. More extensive information regarding the mechanical properties of CNTs is found in [Qian et al., 2002].

While having an extraordinary stiffness, CNTs are very resilient due to their high aspect ratio and thinness as well as the ability of carbon atoms to rehybridize after bending [Krueger, 2010]. Another interesting point about CNTs is that in spite of their exceptional strength, they are very light in weight. For example, they are six times lighter than stainless steel yet 20 times stronger [Kumar and Ando, 2010a]. Therefore, CNTs are considered ideal reinforcing agents for composite materials [Breuer and Sundararaj, 2004].

2.6 Applications of CNTs

The extraordinary properties of CNTs make them applicable in different fields including electronic, field emission, energy storage, sensor, mechanical reinforcement, and biological applications [Endo et al., 2008]. For example, CNTs are used in atomic force microscopy (AFM) to sharpen the tip, obtain better resolution, and avoid damages to the surface of the sample because of their suitable shape (i.e. large proportion of length to diameter) and their mechanical resistance against the bending strain [Krueger, 2010].

The field emission property of CNTs (i.e. their ability to emit electrons from their tips when placed in an electric field parallel to their axis) makes them useful in the development of flat panel displays [Poole and Owens, 2003]. Moreover, the electric conductivity of semiconducting CNTs is influenced by heating and applying an external field which is called the field effect. This property of semiconducting CNTs can be used in the design of field effect transistors [Krueger, 2010]. CNTs can also have applications in battery technology because they can provide efficient means to store lithium which is a charge carrier in some batteries or hydrogen which is used in fuel cells [Poole and Owens, 2003].

Since many properties of CNTs are sensitive to their environment such as mechanical pressure and concentration of certain molecules, these materials can be used as physical and chemical
sensors [Li et al., 2008; Sinha et al., 2006; Stampfer et al., 2006; Yun et al., 2007]. For example, the Raman spectrum of a CNT changes as a result of mechanical strain which can be used to make sensors that detect strain [Krueger, 2010]. Another example is the sensitivity of CNTs conductivity to the presence of certain gases such as NO\(_2\) which is used to design highly sensitive small sensors to detect the kind and concentration of substances in the environment [Krueger, 2010].

CNTs have also been used to increase the strength of materials. For example, the tensile strength of steel was calculated to increase 7 times by adding 30 wt % of CNTs to it [Poole and Owens, 2003]. Additionally CNTs have a huge potential in biomedical applications. They can be used as imaging contrast agents for optical labeling and magnetic resonance imaging [Harrison and Atala, 2007], as drug and gene delivery systems [Bianco et al., 2005], in cancer therapy [Mahmood et al., 2012], in regenerative medicine [Tran et al., 2009], and tissue engineering [Harrison and Atala, 2007] including bone applications, the interest of the present study.

However, the first step towards applying CNTs in any application including orthopedic materials is to evaluate their cytotoxicity and understand their effects on the cells present in the tissue of interest. In the following sections, therefore, the techniques usually used for evaluating the cytotoxicity of nanomaterials including CNTs are briefly presented and then to better understand the effects of CNTs on the bone cells, bone tissue is briefly described.

### 2.7 Assays for evaluating cytotoxicity in vitro

Nanotoxicology is a multidisciplinary field that requires cooperation of material, biology, and toxicology sciences in order to understand the interactions of nano and bio phases (for review see [Kunzmann et al., 2011; Lewinski et al., 2008; Morimoto et al., 2010; Ober dorster et al., 2005b; Smart et al., 2006; Warheit, 2010]).

The first step towards understanding how a material will react in body often involves in vitro studies which are less expensive, easier to control and reproduce than in vivo ones and allow for testing specific biological impacts under controlled conditions [Ober dorster et al., 2005a; Sutter, 1995]. In cell culture experiments, adherent cells (e.g. fibroblasts and osteoblasts) firstly attach to the surface on which they are seeded (e.g. polystyrene (PS) culture plates) by
Physicochemical interactions between cells and materials including ionic and van der Waals forces [Anselme, 2000]. Afterwards cell membrane receptors called integrins bind to specific proteins adsorbed on the surface from cell culture media, develop contact points (i.e. focal adhesion points) and initiate a signaling pathway that eventually regulates cell growth and differentiation [Anselme, 2000]. Indeed, integrins binding to their ligands cause a sequential formation of certain proteins inside the cell which leads to the organization of actin filaments that are linked to the nucleus [Anselme, 2000].

Once a material with cytotoxic properties enters the cell environment, it causes cell function impairment, cell detachment, and cell death (i.e. apoptosis or necrosis), or changes in the deoxyribonucleic acid (DNA) sequence or ribonucleic acid (RNA) expression levels [Hillegass et al., 2010]. Cytotoxicity is usually measured by colorimetric or fluorescent assays that detect cell viability or death after a certain incubation time [Kroll et al., 2009]. Many of the assays that measure cell viability are based on assessing the proper functioning of different cell organelles such as mitochondria or lysosomes [Hillegass et al., 2010; Kroll et al., 2009]. Cell death assays are mostly based on the fact that high cytotoxicity results in cell death either by causing drastic cell membrane damages or by apoptosis which is the programmed cell death that eliminates non-functional or injured cells [Hillegass et al., 2010; Kroll et al., 2009]. A number of techniques for cytotoxicity testing of nanomaterials and their principles are summarized in Table 2.2.

Specific characteristics of nanomaterials may result in interferences with cytotoxicity assays (for review see [Kroll et al., 2009]). For example, nanomaterials possess large surface area which may result in the adsorption of dyes and substrates used in various cytotoxicity assays [Casey et al., 2007; Worle-Knirsch et al., 2006]. Moreover, the high adsorption capacity of nanomaterials results in the adsorption of proteins upon entering biological environments. This phenomenon can either directly affect the results of cytotoxicity assays if the method is using proteins [Veranth et al., 2007] or indirectly induce false outcomes by adsorption of nutrients from culture medium [Guo et al., 2008]. Therefore, a detailed and complete material characterization is essential to better understand how different properties affect biological responses of these materials [Oberdorster et al., 2005a; Warheit, 2008]. Furthermore, different
cytotoxicity techniques and adequate controls must be employed to achieve reliable results and conclusions. More detailed information about the cytotoxicity techniques used in the present study is presented in chapters 3 and 5.

Table 2.2 Assays for testing cytotoxicity in vitro.
[Hillegass et al., 2010; Kroll et al., 2009; O’Brien et al., 2000].

<table>
<thead>
<tr>
<th>Cytotoxicity assays</th>
<th>Detection</th>
<th>Assay basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT/MTS</td>
<td>Colorimetric detection of mitochondrial enzymatic activity</td>
<td>- Reduction of a tetrazolium dye to a formazan in viable cells with intact mitochondria</td>
</tr>
<tr>
<td>Alamar blue assay</td>
<td>Fluorimetric detection of metabolically active cells</td>
<td>- Ability of viable cells to convert resazurin to the fluorescent resorufin product proportional to the number of metabolically active, viable cells</td>
</tr>
<tr>
<td>Neutral red (NR) assay</td>
<td>Colorimetric detection of intact lysosomes</td>
<td>- Uptake of the NR dye through cell membrane into the cytosol and its accumulation in the intact lysosomes of the viable cells</td>
</tr>
<tr>
<td>Cell Titer-Glo® Luminescent Cell Viability Assay</td>
<td>Luminescence detection of amount of ATP (adenosine triphosphate) from viable cells</td>
<td>- Loss of the ability to synthesize ATP in cells with lost membrane integrity and viability</td>
</tr>
<tr>
<td>Colony assay</td>
<td>Detection of colony-forming efficiency (CFE)</td>
<td>- Formation of cell colonies with different sizes and morphologies as a result of decreased or increased survival and proliferation over extended periods of time</td>
</tr>
<tr>
<td>Lactate dehydrogenase assay (LDH)</td>
<td>Colorimetric detection of cell membrane integrity by measuring the LDH release</td>
<td>- Conversion of a tetrazolium salt to a formazan as a result of LDH leakage to the extracellular environment from cells with damaged membranes</td>
</tr>
<tr>
<td>TUNEL assay</td>
<td>Detection of apoptosis</td>
<td>- Labeling of the fragmented ends of DNA due to apoptosis resulting in biotinylated dUTP at the 3'‑OH end</td>
</tr>
<tr>
<td>Apostain assay</td>
<td>Detection of apoptosis</td>
<td>- Detection of single-stranded DNA in condensed chromatin in apoptotic cells by apostain antibody</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Fluorimetric detection of caspase-3 activity (apoptosis marker)</td>
<td>- Measuring the cleavage of caspase-3 present in the terminal apoptosis stages</td>
</tr>
<tr>
<td>Annexin V/propidium iodide (PI)</td>
<td>Fluorimetric detection of phosphatidylinerine exposure/PI-staining of DNA</td>
<td>- Binding of Annexin V to phosphatidylinerine on the outer surface of the plasma membrane in apoptotic cells - Nuclear staining by PI in cells with lost membrane integrity (late apoptotic or necrotic cells)</td>
</tr>
</tbody>
</table>

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyoxapheny1)-2-(4-sulfophenyl)-2H-tetrazolium; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
2.8 Bone tissue

2.8.1 Bone function and structure

Bone is a highly vascularized, innervated, and mineralized conjunctive tissue which supports the body, protects the internal organs, makes the movement possible, stores minerals and growth factors, maintains calcium and phosphate homeostasis, and is the site of hematopoiesis [Marieb and Katja, 2007]. Bones are classified based on their shape into long, short, flat and irregular categories [Marieb and Katja, 2007]. Structure of a typical long bone consists of a long shaft, called diaphysis, and two expanded ends called epiphysis (Figure 2.6) [Bergeron, 2010; Marieb and Katja, 2007]. Apart from this classification, bones are either compact (or cortical, 5 to 30 % porous) or spongy (or trabecular, 30 to 90 % porous) [Bergeron, 2010; Marieb and Katja, 2007]. Compact bone is the external smooth and solid looking part arranged concentrically in units called osteon around the haversian canals which contain the blood vessels and nerves [Marieb and Katja, 2007]. These canals are connected to the lacunae, the spaces which constrain osteocytes, the cells embedded in the bone matrix, through thin canals called canaliculi [Marieb and Katja, 2007]. Mechanical support and protection are the main responsibilities of the compact bone. Spongy bone restricts the cavities containing the bone marrow [Marieb and Katja, 2007] and is responsible for the metabolic activity of the bone [Marquis et al., 2009].
2.8.2 Bone matrix components

Bone extracellular matrix

Bone tissue contains bone cells and the ECM. Bone ECM is composed of an organic phase (22 %) also called the osteoid which gives bone its flexibility and an inorganic or mineral phase (70 %) which provides bone’s structure [Marquis et al., 2009]. The remaining 8 % is made of water [Marieb and Katja, 2007]. The major part (90 %) of the organic ECM consists of fibrillar proteins especially collagen, mostly type I [Fernandez-Tresguerres-Hernandez-Gil et al., 2006a]. The non-collagenous proteins present in the bone matrix (10 %) are mainly (a) cell attachment proteins such as fibronectin and bone sialoprotein (BSP), (b) proteoglycans which contain glycosaminoglycan side chains such as heparan sulfate, (c) proteins with γ-carboxyglutamic acid such as osteocalcin (OC), (d) glycoproteins such as osteonectin and...
ALP, (e) the proteins which originate from plasma such as albumin, and (f) the growth factors such as TGF-β superfamily [Fernandez-Tresguerres-Hernandez-Gil et al., 2006a].

The inorganic phase mainly consists of calcium and phosphate in the form of HAP crystals (Ca_{10} (PO_{4})_{6} (OH)_{2}) [Fernandez-Tresguerres-Hernandez-Gil et al., 2006a]. Mineralization involves the nucleation of HAP crystals and their deposition between the collagen fibrils [Nudelman et al., 2010; Ottani et al., 2002]. The inorganic phase also contains sodium, magnesium, potassium, phosphorous, citrate, fluoride, and chloride and acts as the major ion-reservoir of the body [Cohen, 2006].

**Bone cells**

Mainly there are three types of differentiated cells in the bone matrix: osteoblasts, osteocytes and osteoclasts. Bone cells originate from two types of stem cells. Osteoblasts and osteocytes originate from MSCs which are fibroblast-like stem cells present in bone marrow capable of differentiating into osteoblasts, adipocytes, and chondrocytes [Abdallah and Kassem, 2008] while osteoclasts originate from hematopoietic stem cells (HSCs) [Del Fattore et al., 2010] (Figure 2.7).

![Figure 2.7](image)

Figure 2.7  Mesenchymal and hematopoietic stem cells give rise to osteoblasts, osteocytes, and osteoclasts respectively.

Figure created using [Servier Medical Art, 2005].
Osteoblasts are mononucleated cells of about 20-30 μm size responsible for bone formation and its mineralization [Fernandez-Tresguerres-Hernandez-Gil et al., 2006a]. In the development of osteoblasts, MSCs become preosteoblasts under the influence of hormones, vitamins and growth factors such as fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and BMPs [Hughes-Fulford and Li, 2011]. Wnt ligand (i.e. a group of secreted proteins involved in different embryonic processes) also plays a crucial role in osteogenic differentiation of MSCs by blocking their adipogenic differentiation [Glass II and Karsenty, 2007]. Preosteoblasts then, become mature osteoblasts which synthesize most of the ECM proteins such as type I collagen and OC, and also express proteins such as ALP necessary for calcification [Cohen, 2006; Marquis et al., 2009]. The osteogenic differentiation therefore requires the activation of genes encoding osteoblastic markers which is regulated by the formation of complexes made of signaling molecules (e.g. phosphorylated small mothers against decapentaplegic (Smad) protein in BMP pathway) and transcription factors like runt-related transcription factor 2 (Runx2), osterix (Osx) and distal-less homeobox 5 (Dlx5) (Figure 2.8) [Cohen, 2006; Holleville et al., 2007; Marquis et al., 2009; Nakashima et al., 2002]. Molecular markers of the early osteogenic differentiation are type I collagen and ALP while OC and mineralization of the ECM are markers of the mature osteoblast phenotype [Cohen, 2006]. Osteonectin, osteopontin, and BSP are other important markers of osteogenic differentiation [Cohen, 2006]. For example, BSP is involved in mineralization [Yang et al., 2010]. As the mineralization of the osteoid progresses, osteoblasts become entrapped in the lacuna as osteocytes [Marquis et al., 2009]. Otherwise, they either become bone lining cells which have flat and thin morphology and are involved in bone remodeling, or they die through apoptosis [Marquis et al., 2009]. Osteocytes are terminally differentiated cells of the osteoblast lineage and the most numerous among bone cells [Marquis et al., 2009]. Osteocytes are located in lacunae near blood vessels that provide the nutrients and oxygen required for their survival and are mainly responsible for controlling the bone remodeling through sensing the mechanical stress [Heino et al., 2009; Krause et al., 2010; Raggatt and Partridge, 2010]. Osteocytes form a network by connecting together through long dendrite-like canaliculi which are the routes of communication between these cells. Osteocytes also communicate with
osteoblasts through these canaliculi as well as transmembrane proteins or integrins which are the links between cells and the ECM [Fernandez-Tresguerres-Hernandez-Gil et al., 2006a].

Figure 2.8  Schematic presentation of MSC differentiation in to adipocytes, chondrocytes, and osteoblasts under the influence of transcription factors. Figure created using [Servier Medical Art, 2005].

Osteoclasts are multinucleated cells of about 100 μm size primarily responsible for the degradation of the bone [Bruzzaniti and Baron, 2006]. They are derived from monocyte/macrophage lineage originated from HSCs [Marquis et al., 2009]. Osteoclastogenesis (i.e. osteoclast differentiation from haematopoietic stem cells), is mainly regulated by two cytokines: (a) macrophage-colony stimulating factor (M-CSF) and (b) receptor activator of nuclear factor kappa beta ligand (RANKL) which is mainly produced by osteoblasts [Yasuda et al., 1998]. RANKL activates the transmembrane ligand RANK (i.e. receptor activator of nuclear factor kappa beta) on osteoclasts membrane. This activation results in the internal structure changes such as actin cytoskeleton rearrangement, in a form of a ring, in osteoclasts and consequently formation of a tight junction between the bone surface and basal membrane of these cells in order to resorb bone [Boyle et al., 2003]. However, osteoblasts can also synthesize osteoprotegerin (OPG), a member of tumor necrosis factor (TNF) family, that inhibits osteoclast differentiation and activation [Stolina et al., 2009].
Bone lining cells may also be involved in the suppression of osteoclastogenesis [Matsuo, 2009].

2.8.3 Bone remodeling

Although it might appear otherwise, bone is a dynamic tissue which undergoes a continuous remodeling throughout life. Each year about 5% of the compact bone and 20% of the spongy bone are renewed by remodeling process during which old bone is removed by osteoclasts and instead new bone is formed by osteoblasts [Fernandez-Tresguerres-Hernandez-Gil et al., 2006b; Matsuo, 2009]. In healthy adults (normally until the age of 50), formation and resorption rates are tightly controlled and balanced in order to keep the bone mass constant [Marieb and Katja, 2007]. In general, bone remodelling comprises 4 phases which are (a) preosteoclast recruitment and bone resorption, (b) reversal, (c) preosteoblast recruitment and bone formation, and (d) termination (Figure 2.9). In fact, it seems that bone remodelling is activated by apoptosis of osteocytes in response to mechanical or hormonal signal (e.g. parathyroid hormone) which will result in osteoclastogenesis due to the reduction of TGF-β secretion by osteocytes [Raggatt and Partridge, 2010]. In response to these signals, osteoblasts cause pre-osteoclast differentiation through a complicated mechanism which involves expression of several osteoclastogenic molecules such as RANKL which all together result in spreading, cytoskeletal organization and proliferation of osteoclast precursors and their differentiation [Raggatt and Partridge, 2010]. Osteoblasts also secret matrix metalloproteinases (MMPs) and degrade the bone surface exposing the adhesion sites (e.g. Arg-Gly-Asp, RGD) needed for osteoclasts atachment (e.g. through alphavbeta3 integrins) [Raggatt and Partridge, 2010]. Then the mature osteoclasts attach to the bone matrix and dissolve the old bone through acidification (proton ATPases pump) and secretion of enzymes such as MMP and cathepsin K [Marquis et al., 2009; Sims and Gooi, 2008]. When the destruction stage is done, mature osteoclasts die through apoptosis and mononuclear cells which might be macrophages and/or bone lining cells show up on the site in order to phagocyte any remaining debris and probably secret factors that help initiate bone formation [Raisz, 1988; Sims and Gooi, 2008]. Mature osteoclasts also secret some molecules such as cardiortropin-1 (CT-1) which may recruit pre-osteoblasts to the site [Walker et al., 2008]. Pre-
osteoblasts differentiate into mature osteoblasts which will fill the destructed site by sensing the changes in physical dimensions of the bone or the composition of the surface [Sims and Gooi, 2008]. Osteoblast differentiation and bone formation are also stimulated by regulatory factors embedded in the bone matrix (e.g. BMPs) that are released during its resorption by osteoclasts [Sims and Gooi, 2008]. Collagen type I and other non-collagenous proteins of the bone matrix will be secreted by osteoblasts followed by HAP incorporation into this matrix and finally mineralization [Raggatt and Partridge, 2010]. When the same amount of bone that was resorbed by osteoclasts is replaced, osteoblasts either go through apoptosis, or become osteocytes or transform into bone lining cells and bone remains in resting state until the next remodeling phase [Raggatt and Partridge, 2010].

Figure 2.9 Bone remodeling involves recruitment and differentiation of osteoclast and osteoblast precursors and also participation of osteocytes and bone lining cells. Figure created using [Servier Medical Art, 2005].

2.8.4 Fracture healing: repair and regeneration

When the bone tissue is injured, it heals through repair or regeneration according to the type of fracture [Al-Aql et al., 2008]. The difference between these processes is that in bone repair,
healing happens in the continuity of the injured tissue, while regeneration healing consists of differentiation of new cells and new bone formation [Al-Aql et al., 2008]. Endochondral bone healing process has four stages: (a) inflammation, (b) cartilage formation, (c) cartilage resorption and primary bone formation, and (d) secondary bone formation and remodeling [Al-Aql et al., 2008]. Briefly, right after the injury and hematoma formation, macrophages along with other inflammatory cells synthesize inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and TNF-α recruiting MSCs to the injured site which in turn will produce some growth factors such as BMPs. MSCs differentiation into chondrocytes is followed by cartilage formation and vascularization. Interference of specific cytokines such as TNF-α will then cause chondrocyte apoptosis and cartilage resorption as well as recruitment of preosteoclasts and their differentiation into osteoclasts. In the next step, in presence of BMPs, osteoblasts, differentiated from preosteoblasts and MSCs, form the bone tissue which is followed by remodeling process and bone marrow formation [Al-Aql et al., 2008; Lauzon et al., 2012; Phillips, 2005]. As mentioned above, several factors are involved in bone tissue development and repair among which BMPs are of great interest due to their high osteoinductive potential [Yamaguchi et al., 2000].

2.8.5 Bone morphogenetic proteins

BMP classification
BMPs are members of TGF-β family that are involved in several cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death [Canalis et al., 2003; Massague, 1998]. In 1965 Dr. Marshall Urist showed induction of new bone formation by implantation of demineralized bone in muscles of rodents [Urist, 1965]. The molecules involved in this phenomenon were later identified as BMPs. To date, about 20 members of the BMPs have been identified and classified into subfamilies based on the similarity of their amino acid sequences and functions: (a) BMP-2/-4, (b) BMP-3, (c) BMP-5/-6/-7/-8, (d) BMP-9/-10, (e) BMP-12/-13/-14 and (f) others subfamilies [Senta et al., 2009]. BMPs are entrapped in ECM [Miller et al., 2000] and are also expressed during different stages of fracture healing. Bone forming cells can synthesize BMP-2,-3,-4,-6,-7,-8 [Van der Horst et al., 2002]. BMP-9 is produced by non-parenchymal cells of the liver [Miller et al., 2000].
2000] and is found in the blood plasma (2-12 ng/mL) [David et al., 2008]. However, a recent study has revealed that hepatocytes are the main cellular source of circulating BMP-9 [Bidart et al., 2012]. BMPs induce MSCs to differentiate towards osteoprogenitors and also enhance osteoblastic differentiation through up-regulation of important osteogenic markers such as Runx2, Osx and Dlx5 [Ducy and Karsenty, 2000]. BMP-2, -4, -6, -7, -9 can induce a complete bone morphogenesis [Bessa et al., 2008]. BMP-2 and BMP-7 also play a role in the proliferation, differentiation and apoptosis of the bone cells [Senta et al., 2009; Wei et al., 2008]. BMP-2, BMP-6, and BMP-9 have been shown to induce significant ALP activity in murine mesenchymal multipotent C3H10T1/2 and also human myoblastic C2C12 cells [Cheng et al., 2003; Kang et al., 2004].

**BMP signaling**

BMPs act on the bone cells by binding to specific receptors located on the cell membrane and activating specific intracellular signaling pathways resulting in the expression of specific genes [Marquis et al., 2009]. BMPs bind to the extracellular domain of a heterotetrameric complex composed of two type I and two type II receptors [Chen et al., 2004a]. Subsequently, the intracellular domains of both receptors approach each other permitting the phosphorylation of the type I receptor by type II receptor [Ross and Hill, 2008].

**BMP receptors**

The transmembrane receptors that bind to BMPs are serine/threonine kinases (i.e. they catalyze phosphorylation of the serine or threonine amino acids in the polypeptide sequence) [Senta et al., 2009]. Based on their structural and functional properties, these receptors are categorized into type I receptors and type II receptors [Massague, 1998]. In the three dimensional structure of the BMPs, there is a part called *wrist* epitope which binds to receptor type I and another part called *knuckle* epitope which binds to receptor type II [Kirsch et al., 2000a; Kirsch et al., 2000b]. Receptors that bind to BMPs can be classified into BMP receptors (BMPR), activin receptors (ActR), and activin receptor-like kinases (ALK) [Chen et al., 2004b]. In fact, six different type I ALK receptors (i.e. ALK1/2/3/4/5/6) and three different type II receptors (i.e. BMPRII, ActRIIA and ActRIIB) bind to BMPs [Massague, 1998]. For example, receptor type I ALK3 is necessary for BMP-2/-4 subfamily for signal
transduction [Lavery et al., 2008]. For example, MC3T3-E1 preosteoblasts have ALK2/3/4/5 but they are deprived of ALK1 and ALK6 [Murakami et al., 2009]. Table 2.3 summarizes most of the known BMPs, their receptors and functions.

### Table 2.3 BMP subfamilies, receptors and functions.

Adapted from [Bessa et al., 2008; Lauzon et al., 2012; Marquis et al., 2009; Senta et al., 2009].

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>BMP</th>
<th>Receptor type I</th>
<th>Receptor type II</th>
<th>Signal</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2/-4</td>
<td>BMP-2</td>
<td>ALK3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Cartilage &amp; bone morphogenesis /heart formation</td>
</tr>
<tr>
<td>BMP-4</td>
<td>ALK3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Cartilage &amp; bone morphogenesis /kidney formation</td>
<td></td>
</tr>
<tr>
<td>BMP-3</td>
<td>BMP-3</td>
<td>ALK4</td>
<td>ActRIIA</td>
<td>Smad2/3</td>
<td>Negative regulator of bone morphogenesis</td>
</tr>
<tr>
<td>BMP-5/-6/-7/-8</td>
<td>BMP-5</td>
<td>ALK3/6</td>
<td>?</td>
<td>Smad1/5/8</td>
<td>Limb development /bone morphogenesis</td>
</tr>
<tr>
<td>BMP-6</td>
<td>ALK2/3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Cartilage hypertrophy /bone morphogenesis /estrogen mediation</td>
<td></td>
</tr>
<tr>
<td>BMP-7</td>
<td>ALK2/3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Cartilage &amp; bone morphogenesis /kidney formation</td>
<td></td>
</tr>
<tr>
<td>BMP-8</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Bone morphogenesis /spermatogenesis</td>
<td></td>
</tr>
<tr>
<td>BMP-9/-10</td>
<td>BMP-9</td>
<td>ALK1/2</td>
<td>BMPRII/ActRIIA/ActRIIB</td>
<td>Smad1/5/8</td>
<td>Bone morphogenesis /development of cholinergic neurons/glucose metabolism</td>
</tr>
<tr>
<td>BMP-10</td>
<td>ALK1/3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Heart morphogenesis</td>
<td></td>
</tr>
<tr>
<td>BMP-12/-13/-14</td>
<td>BMP-12</td>
<td>ALK3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Ligament &amp; tendon development /sensory neurons development</td>
</tr>
<tr>
<td>BMP-13</td>
<td>ALK3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Cartilage development &amp; hypertrophy</td>
<td></td>
</tr>
<tr>
<td>BMP-14</td>
<td>ALK3/6</td>
<td>BMPRII/ActRIIA</td>
<td>Smad1/5/8</td>
<td>Chondrogenesis/angiogenesis</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>BMP-11</td>
<td>ALK4</td>
<td>ActRIIA/ActRIIB</td>
<td>Smad2/3</td>
<td>Axial skeleton patterning /eye development/pancreas development/kidney formation</td>
</tr>
<tr>
<td></td>
<td>BMP-15</td>
<td>ALK6</td>
<td>BMPRII</td>
<td>Smad2/3</td>
<td>Ovary physiology</td>
</tr>
</tbody>
</table>

### Smad signaling pathway

Different Smads are involved in the BMP induced Smad signaling: (a) the receptor-regulated Smad proteins (R-Smads) of which different types including Smad1, Smad5, Smad8 are
present in mammalian cells [Bessa et al., 2008], (b) the common-partner Smad (Co-Smad) or Smad4, and (c) the inhibitory Smads (I-Smads) containing Smad6 and Smad7 [Senta et al. 2009] (Figure 2.10).

After binding of BMPs to the transmembrane receptors, activation of type I receptor by type II receptor induces phosphorylation of R-Smad which will then form a complex with Co-Smad [Senta et al. 2009]. This complex translocates to the nucleus and in collaboration with transcription factors (TF) such as Runx-2 regulates the expression of genes encoding early (collagen type I, Runx2, Osx, osteopontin, and osteonectin) or late (OC and BSP) osteogenic markers [Ross and Hill, 2008]. I-Smads (Smads 6 and 7) inhibit the Smad signaling through degradation of Smad proteins by Smad ubiquitin regulatory factors (Smurfs) [Ross and Hill, 2008] or through prevention of R-Smad phosphorylation and formation of R-Smad/Co-Smad complex [Von Bubnoff and Cho, 2001].

BMP and mitogen activated protein kinase pathways

In addition to Smad pathway, BMPs can induce gene expression through the activation of mitogen activated protein kinase (MAPK) signaling pathway which has three cascades: (a) extracellular signal-regulated kinase (ERK), (b) c-Jun N-terminal kinase (JNK) and (c) p38 MAPK [Guicheux et al., 2003; Jun et al., 2010; Senta et al., 2009] (Figure 2.10).

This pathway is activated when the TGF-β activated kinase 1 (TAK1) is activated by type I receptor possibly by X-chromosome-linked inhibitor of apoptosis (XIAP). Formation of a complex containing TAK1 and TAK binding protein 1 (TAB1) activates different cascades of this pathway [Senta et al., 2009].
2.9 CNTs in orthopedic applications

In the case of bone damages of certain size caused by fracture, or bone diseases, bone tissue is not capable of proper self-repair and there is a need to fill the defect by bone substitutes during surgery to help and improve the repair. Unique properties of nanomaterials including CNTs are advantageous for the design of improved bone substitutes that support and enhance cell and tissue growth [Christenson et al., 2007; Haniu et al., 2012]. Due to their exceptional mechanical strength, flexibility, and low density, CNTs are ideal for production of materials that should be both highly strong and light in weight. Applying CNTs in the biomaterials used as bone substitute is expected to improve their mechanical properties [Usui et al., 2008]. Summaries of a number of studies that have analyzed the mechanical properties and bone cell/tissue response of the composites containing synthetic polymers and CNTs, natural polymers and CNTs, and HAP and CNTs are shown in APPENDIX A, B, and C respectively. For example, CNTs at concentrations as low as 0.05 wt % and up to 10 wt % have been shown to improve the mechanical properties of natural or synthetic polymeric scaffolds [Moon et al.,
2005; Shi et al., 2005]. An increase has been reported in Young’s modulus and fracture toughness of PMMA, a polymer that is used in orthopedic surgeries as filling cement, when it is reinforced with CNTs [Marrs et al., 2006].

CNTs are referred to as similar to the ECM in several aspects such as high flexibility and elasticity, large surface area, and high degree of porosity [Tonelli et al., 2012]. CNTs can be used in fabrication of 3D scaffolds mimicking the natural ECM [Hirata et al., 2009; Hirata et al., 2010] which promote osteoblastic differentiation [Tu et al., 2007]. SWCNTs have also good potential to be used as scaffolds for the nucleation and mineralization of HAP crystals because of the similarity of their diameter size to that of triple helix collagen fibrils [Akasaka et al., 2006; Zanello et al., 2006; Zhao et al., 2005]. It is also possible to functionalize CNTs with proteins of ECM [Cai et al., 2010] or peptides [Villa et al., 2011] to increase their biocompatibility and also make them as similar as possible to the ECM [Tonelli et al., 2012].

In addition, protein adsorption which is a crucial parameter in the attachment of cells to biomaterials [Webster et al., 2001] is enhanced on nano-scale roughness and topography (i.e. irregularities less than 100 nm) [Vandrovcova and Bacakova, 2011]. Furthermore, these proteins are adsorbed on nano surfaces with their natural physiological geometrical conformation [Vandrovcova and Bacakova, 2011]. In addition, due to their nanostructure, large surface area, high surface energy, crystalline structure, and the porosity of the network structure, culture medium can easily be soaked into the dense meshwork nanostructure formed by the CNTs resulting in the adsorption of proteins such as fibronectin and vitronectin in the serum which influence the cellular responses [Aoki et al., 2007].

2.10 Effect of CNTs on the bone forming cells

It is of great importance to study the effect of CNTs on the bone cells attachment, viability, proliferation, differentiation, and function to ensure their biocompatibility with bone cells prior to using them in the design of biomaterials. Although there have been several studies on the influence of CNTs on osteoblastic cells, most of them have evaluated the effect of composites containing CNTs. Therefore, it is difficult to identify the effects that are directly caused by CNTs. A number of studies that have investigated the direct effect of CNTs on bone
cells are summarized in Table 2.4 which mostly have shown good biocompatibility of CNTs to osteoblastic cells in vitro. Moreover, bone tissue biocompatibility of CNTs has also been evaluated in vivo [Li et al., 2012; Mendes et al., 2010; Usui et al., 2008; Wang et al., 2007; Wang et al., 2008]. Furthermore, few studies have investigated the effect of BMP-2 and CNTs combination on the ability of bone forming cells to differentiate in vitro as well as bone formation in vivo which are summarized in Table 2.5.
Table 2.4  Effect of CNTs on the bone cells.

<table>
<thead>
<tr>
<th>Cell</th>
<th>CNT</th>
<th>Description</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat osteosarcoma cells (ROS 17/2.8)</td>
<td>As-produced and functionalized (nitric acid, poly-m-amino benzene sulfonic acid and polyethylene glycol (PEG)) SWCNTs &amp; MWCNTs</td>
<td>CNTs (100 μg/mL) sonicated in water or ethanol 95% for 2 h then sprayed onto preheated glass cover slips</td>
<td>- No effect on the growth and mineralization of osteoblasts cultured on electrically neutral CNTs</td>
<td>[Zamello et al., 2006]</td>
</tr>
<tr>
<td>Human osteoblastic cell line (hFOB 1.19 ATCC CRL-11372)</td>
<td>Purified CVD MWCNTs (CoO/MgO as catalysts)</td>
<td>Polysulfone ((C_7H_7O_3S)_n) dispersed in methylene chloride, poured onto a petri plate and then covered with CNTs</td>
<td>- Good biocompatibility of CNTs, similar to that of polysulfone currently used in medicine</td>
<td>[Chlopík et al., 2006]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>CVD MWCNTs (d: 5-20 nm l: 20-40μm), purified by HCl (98 wt %)</td>
<td>CNTs dispersed in D-H2O, filtered on porous polycarbonate membrane (PC) Scaffolds placed in PS dishes for cells seeding</td>
<td>(+) - Excellent adherence and proliferation of the cells on CNTs, with extended morphology and growth of numerous filopodia</td>
<td>[Aoki et al., 2006]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>CVD MWCNTs (d: 13-53 nm, grown on quartz substrate with Fe catalysts)</td>
<td>MWCNTs constructs placed in 24-well plates for cell seeding</td>
<td>(+) - Higher metabolic activity (measured by MTT assay) of the cells grown on MWCNT constructs after 7 days</td>
<td>[Mwenifumbo et al., 2007]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>SWCNTs &amp; MWCNTs purified by HCl</td>
<td>CNTs dispersed in distilled H2O filtered on porous PC Scaffolds placed in PS dishes for cell seeding</td>
<td>(+) - Higher adsorption of proteins on SWCNTs than on MWCNTs and other substrates - Enhanced cell proliferation and growth on CNTs with extended morphology</td>
<td>[Aoki et al., 2007]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>Purified SWCNTs, non-purified laser ablation SWCNTs (Ni-Co as catalyst), HiPco SWCNTs (Ni-Y as catalyst)</td>
<td>CNTs suspended in ethanol (100 μg/mL) placed on PS culture dish to dry</td>
<td>(+/-) - Non-toxicity of the SWCNT films to osteoblasts in the same range as Ti6Al4V alloy (standard material used for implants) - Significant reduction of metabolic activity only by arc discharge SWCNT with the highest impurity content</td>
<td>[Kalbacova et al., 2007]</td>
</tr>
<tr>
<td>Cell</td>
<td>CNT</td>
<td>Description</td>
<td>Results</td>
<td>Ref.</td>
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<td>------------------------------------------</td>
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<tr>
<td>Mouse primary osteoblasts</td>
<td>CVD SWCNTs (d &lt; 2 nm), DWCNTs (d &lt; 5 nm), and MWCNTs (d &lt; 10 nm) with the same mean length of 5-15 μm, functionalized by sulfuric and nitric acids</td>
<td>CNTs suspended in α-MEM supplemented with 10% FBS (up to 100 μg/mL)</td>
<td>(-) - Reduction of cell viability and inhibition of mineralization by CNTs in a dose-dependent manner - Reduction of Runx-2 and Collagen-I protein expression by CNTs</td>
<td>[Zhang et al., 2007]</td>
</tr>
<tr>
<td>Rat primary calvariae osteoblastic cells and murine preosteoblasts (MC3T3-E1)</td>
<td>HiPco SWCNTs purified by HCl</td>
<td>SWCNTs dispersed using sodium dodecyl sulfonate (SDS) deposited on multicellulose ester membrane (MCE) and placed in culture plates</td>
<td>(-/+ - Stimulation of the synthesis of ECM, thereby enhancing bone tissue histogenesis by limited internalization of SWCNTs by cells and promotion of the release of growth factors (by the dead cells)</td>
<td>[Tutak et al., 2009]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>Arc discharge SWCNTs (90% pure, d: 0.9-1.5 nm, l: 2-3 μm) CVD MWCNTs (98% pure d: 5-20 nm, l:20-40 μm)</td>
<td>CNT scaffolds made by vacuum filtration of the dispersed CNTs slurry onto porous PC</td>
<td>(+ - Full growth of the cells with numerous fine filopodia extended far from cell edge on CNT scaffolds - Increased protein adsorption, cell proliferation and ALP activity as follows: SWCNTs &gt; MWCNTs &gt; graphite)</td>
<td>[Watari et al., 2009]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>Arc discharge SWCNTs (95% pure, d: 0.8-2.5 nm) and MWCNTs (d:30 nm, 98% pure)</td>
<td>CNTs dispersion in 99.5% ethanol (5 μg/mL for SWCNTs 10 μg/mL for MWCNTs) placed on PS dish to dry until 0.5 and 5 μg/cm²</td>
<td>(+ - Best cell proliferation on SWCNTs thin films at low concentration of serum (1%)</td>
<td>[Akasaka et al., 2010]</td>
</tr>
<tr>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>Arc discharge SWCNTs (d: 0.8-2.5 nm &gt;95% pure) CVD MWCNTs (d: 20-40nm in 98% pure)</td>
<td>CNTs dispersion in 99.5% ethanol poured onto a culture dish and dried at RT until 0.5 μg/cm²</td>
<td>(+ - No cytotoxic effect of CNTs - Higher viability of the cells cultured in the MWCNT coated dish than those in the culture dish - Higher vinculin expression and cell adhesion on the rough and curled nanostructure of MWCNTs - No effect of the direct contact of CNTs on the ALP activity of cells)</td>
<td>[Matsuoka et al., 2010]</td>
</tr>
</tbody>
</table>

α-MEM: minimum essential medium alpha; FBS: fetal bovine serum
Table 2.5  Effect of CNTs and BMP-2 combination on the bone cells and tissue.

<table>
<thead>
<tr>
<th>Cell/animal</th>
<th>CNT</th>
<th>Description</th>
<th>Results</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Mouse osteoblast progenitors harvested from calvariae</td>
<td>Chitosan functionalized SWCNTs, acid-oxidized SWCNTs, and surfactant-free pristine SWCNTs</td>
<td>3D scaffolds made by SWCNTs with and without BMP-2 (100 ng/mL)</td>
<td>- Higher level of OC in the cells cultured on the SWCNTs with or without BMP-2, as compared to the cells cultured on the tissue culture plastic plates</td>
<td>[Tu et al., 2007]</td>
</tr>
</tbody>
</table>
| Male ddy mice                        | CVD MWCNT (d:80nm, l:10-20μm, 98 % pure) | Infusion of MWCNTs into the subperiosteal pocket in the skull and tibial bone defects Pellets of MWCNT/collagen/rhBMP-2 (500 μg/2 mg/1 μg/mL) implanted into the dorsal muscles | - Good bone tissue biocompatibility of MWCNTs revealed by no strong inflammation and no influence on bone even when placed in contact with it  
- Integration of MWCNTs into the bone tissue  
- Acceleration of ectopic new bone formation in response to rhBMP-2 with MWCNTs | [Usui et al., 2008] |
| Mouse myoblastic cells (C2C12) and Balb/c type mice | MWCNT functionalized by nitric acid | Construction of a porous 3D scaffold from MWCNTs and chitosan, dispersion of MWCNTs (900 mg) in 15 mL of chitosan solution Placement of cylindrical monoliths into 24-well plates for cell seeding | - Biocompatibility of the MWCNT/chitosan scaffolds for adhesion, spreading, proliferation and viability of preosteoblastic cells.  
- Good in vivo biocompatibility of the scaffold revealed by no chronic inflammation during the whole implantation period  
- Bone tissue regeneration after the 3 weeks, with significant scaffold structure degradation (up to 80 %), and replacement by cells | [Abaratgei et al., 2008] |
| Human embryonic kidney epithelial cells (HEK293) and cervical carcinoma epithelial cells (C33A) | Carboxylated SWCNTs (Sigma Aldrich, d:1.4-1.6 nm, l:0.5-1.5 μm) | Carboxylated SWCNTs dispersed in cell culture medium | - Cell cycle arrest at G1/S transition and inhibition of cell proliferation by suppressing BMP signaling pathway | [Mu et al., 2009] |
| Human osteosarcoma cell line (Saos-2) | CVD MWCNTs (d:90 nm) purified by HCl | Seeding cells on compacts made from MWCNTs or graphite (GP) with or without immersing in rhBMP-2 (500 ng/mL) | - More protein adsorption by MWCNT compacts than GP compacts.  
- Higher ALP per unit cell on MWCNTs compared to GP and control which increased after the adsorption of BMP-2  
- Increased osteonectin, osteopontin and OC gene expression on MWCNTs after the adsorption of BMP-2 | [Li et al., 2009] |

rhBMP: recombinant human BMP
<table>
<thead>
<tr>
<th>Cell/animal</th>
<th>CNT</th>
<th>Description</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse primary osteoblasts and M-CSF-supported bone marrow cells (BMMφ) and ddy mice</td>
<td>MWCNTs (80 nm, l: 8-10 μm) and MWCNTs (d:150 nm, l: 8-10 μm)</td>
<td>In vitro: MWCNTs suspension in PBS plus 0.1% carboxymethyl-cellulose (5 and 50 μg/mL) CB as control In vivo: Pellets of MWCNT/collagen/rhBMP-2 (500 μg/2 mg/5 μg) Pellets without MWCNTs as control Implantation of the composites in the dorsal musculature</td>
<td>In vitro: - No effect of MWCNTs (50 μg/mL) on osteoblasts growth - Inhibition of BMMφs proliferation by 50 μg/mL of MWCNTs - Inhibition of osteoclast formation by 5 μg/mL MWCNTs in co-culture of osteoblasts and bone marrow cells treated with 1α,25(OH)2D3 (10⁻⁸ M) - No effect of 5 μg/mL MWCNTs on RANKL, OPG, and MCSF mRNAs expression in osteoblasts treated with 1α,25(OH)2D3 In vivo: - Lower number of osteoclasts in the ectopic bones at 2 weeks with MWCNTs - Higher bone mineral density of the rhBMP-2/collagen/MWCNT group after 3 months indicating decreased number of osteoclasts and inhibition of ectopic bone resorption</td>
<td>Narita et al., 2009</td>
</tr>
<tr>
<td>Human mesenchymal stem cells (hMSCs)</td>
<td>Ultra pure MWCNTs functionalized by PEG groups</td>
<td>MWCNTs dispersed in milliQ water (10 mg/mL) and sprayed onto preheated coverslips with and without adding BMP-2 (150 ng/mL) in osteogenic medium</td>
<td>In vitro: - No cytotoxicity of MWCNTs (100 μg/mL) at 72 h - No significant difference between cell viability on MWCNT-PEG-coated and plain or PEG-coated coverslips - Less cell attachment and viability on carboxylated MWCNT than MWCNT-PEG - Acceleration of the hMSCs differentiation into osteocyte phenotype by MWCNT-PEG even without BMP-2</td>
<td>Nayak et al., 2010</td>
</tr>
<tr>
<td>Human adipose-derived MSCs (hASCs) and male ddy mice</td>
<td>CVD MWCNTs (d:90 nm, curled) Purified by HCl</td>
<td>In vitro: cells cultured on compacts made of MWCNTs or graphite (GP) with and without immersing in rhBMP-2 (500ng/mL) In vivo: implants placed aseptically in the left dorsal muscle pouch</td>
<td>In vitro: - More protein adsorption on MWCNTs compacts than GP - Better cell attachment and proliferation on MWCNTs - Induction of osteogenic differentiation of hASCs by MWCNTs through higher expression of ALP, Runx2, and Collagen I In vivo: - More new bone formation and integration of MWCNTs into new bone tissue at three weeks</td>
<td>Li et al., 2012</td>
</tr>
<tr>
<td>Mouse myoblastic cells (C2C12)</td>
<td>Carboxylated MWCNTs (inner d:5-12 nm, outer d: 30-50 nm, 1:0.5-2 μm)</td>
<td>Dispersion of MWCNTs (1 mg/ml) in distilled water (up to 100 μg/mL)</td>
<td>- Acceleration of myogenic differentiation under the differentiation environment and inhibition of apoptosis by MWCNTs through positively regulating functions of bHLH (basic helix loop helix) proteins (which promote myogenic differentiation) and decreasing expression of Id (inhibitor of DNA-binding/differentiation) proteins (negative regulators) - Decrease of BMPRI phosphorylation by MWCNTs through binding to BMPRII</td>
<td>Zhang et al., 2012</td>
</tr>
</tbody>
</table>
CHAPTER 3 Induction thermal plasma process modifies the physicochemical properties of materials used for carbon nanotube production, influencing their cytotoxicity

Avant-propos

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Date d’acception: September 4 2012
Revue: Nanotoxicology (Informa health care)

Titre français: Un procédé plasma thermique inductif modifie les propriétés physico-chimiques des matériaux utilisés pour la production de nanotubes de carbone, influençant leur cytotoxicité

Contribution to the document:

This article contributes to the thesis by developing a hypothesis that the materials used for SWCNT synthesis have cytotoxic effects per se and the process used for SWCNT synthesis can change the physico-chemical properties and cytotoxicity of the feedstock materials, especially catalysts. Here the objective was to identify the most cytotoxic materials used for
SWCNT synthesis by RF induction thermal plasma process in order to minimize the health risks related to these materials either being considered as the feedstock that is exposed to workers in laboratories during the synthesis process or as the impurities present in the final product. This step provided the opportunity to obtain important information about the properties of the carbon source and catalysts used in this process and the impact of the plasma treatment on these properties which allowed us to better understand the observed cellular effects of these particles and be able to recognize the origin of the materials cytotoxicity. Furthermore, studying and comparing the cytotoxicity of the catalysts that can be used for synthesis of SWCNTs allowed for establishing proper handling methods to avoid health risks when applying the most harmful ones and also distinguish better catalyst options to be examined for SWCNT production.

Yasaman Alinejad performed the synthesis and cytotoxicity evaluation experimental work, the statistical analyses, and drafting the manuscript under the supervision of Prof. Nathalie Faucheux and Prof. Gervais Soucy.

3.1 Résumé

L'effet du procédé RF-plasma thermique inductif (RFITP) sur la cytotoxicité des matériaux utilisés pour la production des nanotubes de carbone mono parois demeure inconnu. Dans cette étude, l'influence du procédé RFITP sur les propriétés physicochimiques et cytotoxiques de catalyseurs commerciaux Co, Ni, Y₂O₃, Mo et le noir de carbone a été étudiée. Les tests cytotoxiques (MTS, de la LDH, rouge neutre, TUNEL) ont révélé que l'effet cytotoxique le plus important sur les fibroblastes murins Swiss 3T3 était obtenu avec le Co commercial. Ce dernier affecte leur viabilité d'une manière dose dépendante dans les 24 h. Les cellules contiennent aussi moins de fibres d'actine. Bien que le procédé RFITP affecte les propriétés de chaque catalyseur (taille, morphologie, chimie), seule la cytotoxicité du catalyseur Ni a été augmentée. Le Ni traité par plasma induit l'apoptose. En comparant les particules de Ni avant et après le procédé RFITP avec des nanoparticules de Ni commerciales il a été observé que ces particules ayant pourtant une surface spécifique similaire ont des cytotoxicités différentes. Fait intéressant, la toxicité des catalyseurs observée n'était pas principalement due à la libération d'ions.

50
3.2 Abstract
The effect of radio frequency induction thermal plasma (RFITP) process on the cytotoxicity of materials used for single-walled carbon nanotube production remains unknown. In this study, the influence of RFITP process on physicochemical and cytotoxic properties of commercial Co, Ni, Y$_2$O$_3$, Mo catalysts and carbon black was investigated. The cytotoxic assays (MTS, LDH, neutral red, TUNEL) revealed the strongest effect of commercial Co on murine Swiss 3T3 fibroblasts affecting their viability in a dose-dependent manner within 24 h. The cells contained also less actin stress fibers. Although RFITP affects the properties of each catalyst (size, morphology, chemistry), only cytotoxicity of Ni catalyst was increased. The plasma treated Ni induced apoptosis. Comparing Ni particles before and after RFITP process with commercial nanoparticles of Ni revealed that the particles with similar surface area have different cytotoxicities. Interestingly, the observed toxicity of the catalysts was not mainly due to the release of ions.

3.3 Introduction
The rapid growth of nanotechnology has resulted in large scale production of nanomaterials including carbon nanotubes (CNTs). These new nanostructures of carbon have the potential to be used in different fields including cancer therapy [Fadel et al., 2010], bone tissue engineering [Zanello et al., 2006], drug delivery [Harutyunyan et al., 2002], microelectronics [Hayamizu et al., 2008], polymers [Ci et al., 2008], and biosensors [Ong and Grimes, 2001], due to their exceptional properties. For example, CNTs are very strong materials with high Young modulus yet good flexibility [Ruoff and Lorents, 1995; Salvetat et al., 1999c; Treacy et al., 1996; Yu et al., 2000]. They have also high thermal and electrical conductivities [Berber et al., 2000; Ebbesen et al., 1996; Ruoff and Lorents, 1995; Salvetat et al., 1999c; Treacy et al., 1996; Yu et al., 2000].

Since their discovery [Iijima, 1991], different techniques have been proposed for CNT production [Ando and Zhao, 2006; Kingston and Simard, 2006; Kumar and Ando, 2010a]. Radio frequency induction thermal plasma (RFITP) is a promising technique for large-scale production of single-walled carbon nanotubes (SWCNTs) [Kim et al., 2007a].
The generation of CNTs is usually based on the evaporation of a carbon source such as graphite [Bethune et al., 1993], hydrocarbons [Kim et al., 2007b], and carbon monoxide [Chiang et al., 2001] along with or in the presence of metallic catalysts [Kim et al., 2009b]. A wide range of metals (individually or as a mixture) including Fe, Ni, Co, Y, Mo, Au, Ag, and Pt are used as catalysts for CNT production in both pure or oxide forms [Ajayan et al., 1993; Dai et al., 1996; Dervishi et al., 2009; Journet et al., 1997; Kiang et al., 1995; Lambert et al., 1994; Moisala et al., 2003; Zhang and Iijima, 1999]. Our previous study demonstrated the synthesis of high quality SWCNTs by RFITP using carbon black (CB) as a carbon source and a ternary mixture of Ni, Co, and Y$_2$O$_3$ as catalysts [Alinejad et al., 2010].

Because CNTs are expected to be widely used in industry [Tejral et al., 2009], their effect on health and their behaviour in biological environments have become the center of attention [Helland et al., 2008; Johnston et al., 2010]. Nanomaterial heterogeneity is one of the most critical issues in nanotoxicological studies. Various physicochemical features of nanoparticles can lead to different toxic potentials [Schulte et al., 2009]. For example, CNTs synthesized by different methods are expected to have different characteristic properties such as length, diameter, wall number, and purity. Therefore, each CNT can potentially have a specific toxicity profile [Hsieh et al., 2012].

Recently, it was reported that there is a considerable potential for nanoparticle exposure in workplaces, especially during transfer, weighing, blending, and cleaning processes [Han et al., 2008; Methner et al., 2007].

To overcome the nanomaterial related health risks, it is necessary to consider the safety of the synthesis process and the toxicity of the materials used for their production. For example, to attain a safe process for CNT production, it is important to use non-toxic feedstock materials (i.e. carbon source and catalysts) because the people currently most affected by nanomaterials are those working in the laboratories [Bello et al., 2009; Bello et al., 2010; Fiorito et al., 2006b; Maynard et al., 2004] who are in direct contact with feedstock materials.

In addition, the remaining feedstock materials, particularly catalysts, will be present in the final CNT product as a fraction of the impurities affecting its toxicity [Kagan et al., 2006; Pulskamp et al., 2007; Worle-Knirsch et al., 2006]. Different post purification techniques used to remove the metallic catalyst content of the CNTs such as acid leaching [Hu et al., 2003;
Kim et al., 2009a], often modify the properties of CNTs which may lead to different biological responses of these materials [Donaldson et al., 2006; Guo et al., 2007; Hsieh et al., 2012]. Therefore, the main objective of this study is to assess the cytotoxicity of the materials often used in CNT synthesis before and after process to identify the most harmful materials to be able to develop proper sampling and handling methods for minimizing the health risks from the very first steps. Because the effect of RFITP process on the cytotoxicity of the metallic catalysts and CB is unknown, both their physicochemical and cytotoxic properties before and after plasma treatment were analysed to find out whether the production process can change the properties that are important in determining the cytotoxicity of the materials. The characterization of particles especially nanoparticles has been reported to be the first necessary step in toxicology studies to be able to justify their biological behaviours [Warheit, 2008; Warheit et al., 2009]. The fundamental physical and chemical properties of a given material appear to change as the particle size decreases to nano-scale range, most of the time resulting in completely new and different properties [Warheit, 2008]. Therefore in this study, the results obtained with the plasma treated catalysts are compared with those of commercial nano-sized Co, Ni, and Y$_2$O$_3$ to better understand the effect of the synthesis process on the cytotoxicity of the materials. For this purpose, neutral red, MTS, and LDH assays were carried out on Swiss 3T3 fibroblasts. Although different cell types have been selected to evaluate the cytotoxicity of nanomaterials [Foucaud et al., 2010; Lira et al., 2011; Wang et al., 2010], in this study, murine Swiss 3T3 fibroblast cell line was used. This cell line is usually applied for standardised toxicological assessment and has been used in several recent studies on toxicology of nanoparticles [Drescher et al., 2011; Travan et al., 2009]. Moreover, 3T3 fibroblasts are often chosen as a model for stromal cells which are found in connective tissue throughout the body [Sohaebuddin et al., 2010]. The filamentous actin (F-actin) and DNA were also labelled in the cells in contact with different types of metallic catalysts and CB to detect changes in the cytoskeleton and chromatin condensation due to apoptosis. Apoptotic cells were then visualized by TUNEL assay. Finally, the toxicity of the ions released from the catalysts was evaluated to determine whether the toxic effects are due to the direct contact with catalysts or to their released ions.
3.4 Materials and methods

3.4.1 Particles
Micron-sized commercial Cobalt (Co), Nickel (Ni), Yttrium oxide (Y₂O₃) and CB as the typical catalysts and carbon source in SWCNT production by RFITP [Kim et al., 2009b] and micron-sized Molybdenum (Mo) as a potential catalyst are named commercial particles and identified by “c” in this study (e.g. commercial Ni is named Ni_c). All commercial particles were received as dry powder from different suppliers indicated in Table 3.1. Plasma treated particles were synthesized from commercial Ni, Y₂O₃, Mo, and CB using the same RFITP synthesis set-up and under the same operating conditions that SWCNTs are produced. They are named plasma treated particles and identified by “p” in this study (e.g. plasma treated Ni is named Ni_p). Detailed information on the RFITP synthesis process has already been published elsewhere [Alinejad et al., 2010; Kim et al., 2007a]. Briefly, the system consists of three parts: (1) an induction plasma torch which generates the plasma discharge from Ar and He. The torch runs by a 40 kW RF power supply operated at an oscillator frequency of 2-5 MHz, (2) a tubular reactor which provides a suitable environment for the formation of precursors and nanomaterial growth, (3) a filtration system where the product is collected. Because pure metallic nanoparticles are self-ignitable in air, synthesized plasma treated particles were passivated by oxygen gas before opening the set-up for particle collection.

Commercial nano-sized Ni, Co, and Y₂O₃ powders purchased from suppliers indicated in Table 1 are named nano-sized particles and identified by “N” (e.g. commercial nano-sized Ni is named Ni_N).

3.4.2 Material characterization
Morphology and size distribution
Materials were analysed by high resolution scanning electron microscope (HRSEM, Hitachi, S4700) equipped with energy dispersive X-ray spectrometer (EDS) to study the morphology and composition of the particles. A suspension of each sample in acetone was prepared using
an ultrasonic tip. Few droplets of the suspension were placed on a silicon wafer to be dried completely. HRSEM-EDS analysis was then performed on the silicon wafers.

A laser diffraction-based particle size analyser (Mastersizer 2000) was used to measure the size distribution of plasma treated particles. Each plasma treated particle was suspended in deionized water using a specified dispersant for each material (i.e. ammonium citrate for Ni₂P, ethylene glycol for Mo₂P, sodium hexameta phosphate for Y₂O₃P and Triton X-100 for CB₂P). Ultrasonic tip was used to improve the dispersion of the particles and also break down immediate agglomeration formed in all the suspensions. Results of the laser diffraction size distribution measurements were confirmed by HRSEM.

**Specific surface area**

Surface area measurements were performed using an automated multipoint Brunauer-Elmer-Teller (BET) apparatus (ASAP 2020, Micromeritics). Nitrogen adsorptions were carried out at 5 different points (p/p₀: 0.05-0.3). Samples were degassed at 110 °C under vacuum for 16-20 h before the analysis. The BET model was used for calculations [Fagerlund, 1973].

**Composition, crystalline structure, and purity**

Chemical composition and crystalline structure of the particles were analysed by X-ray diffraction analyzer (XRD, PANALYTICAL-X'Pret ProMPD, Cu-Kα, λ=1.54Å). Inductively coupled plasma mass spectroscopy (ICP-MS, Elan DRC 2, Perkin Elmer) was used to identify the metallic composition of different catalysts as well as any trace of metallic impurities in the samples. The samples were treated with concentrated HCl/HNO₃ (3:1) mixture and were allowed to react for 10-15 min at room temperature (RT). Then the samples were heated by microwave for 15 min to reach 210 °C and maintained for 45 min at this temperature and eventually cooled down to RT and then diluted for final ICP-MS measurement.

Carbon elemental analysis was performed on CB samples to determine their C content using a LECO SC-632 elemental analyser.
Surface chemistry

X-ray photoelectron spectroscopy (XPS) was performed on the samples using AXIS ULTRA\textsuperscript{DLD} spectrometer equipped with a monochromatic Al-K\textalpha
textsuperscript{\textalpha} (hv = 1 486.69 eV) source at 225 W to detect the elements present within approximately the outermost ~10 nm of the particle surface. Photoelectrons were collected at an angle of 52.5°. Energy resolution of the system (i.e., source and analyser) was set to 0.3 eV. The elemental composition of the analysed surface areas was obtained from survey spectra collected at pass energy of 160 eV.

3.4.3 Cell culture

Murine Swiss 3T3 fibroblasts were purchased from ATCC-CCL 92\textsuperscript{TM} (USA). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, USA) supplemented with 10 % (v/v) of Fetal Bovine Serum (FBS) containing 1 % (v/v) of penicillin/streptomycin solution (10 000 units of penicillin and 10 000 \textmu g/mL streptomycin, Invitrogen) under a humidified 5 % CO\textsubscript{2} atmosphere at 37 °C. Cell passage was performed using 0.25 % Trypsin-EDTA 1x (Invitrogen). For experimental purpose, cells were seeded in 24-well plates (5000 cells/cm\textsuperscript{2}) and allowed to attach and reach 40 % or 80 % of confluence before treatment with materials.

3.4.4 Cytotoxicity assays

Materials were sterilised in an oven at 180 °C for 3 h. Suspensions of the materials were made in DMEM without phenol red supplemented with 5 % (v/v) FBS. Concentrations were based on the ratio of each particle in the SWCNT synthesis feedstock [Kim et al., 2009b]. Concentrations for different particles were as follows: Co\textsubscript{C} (0.0001-0.026 mg/mL), Ni\textsubscript{C} (0.01-0.06 mg/mL), Mo\textsubscript{C} (0.02-0.1 mg/mL), Y\textsubscript{2}O\textsubscript{3-C} (0.02-0.12 mg/mL), Ni\textsubscript{P}, Mo\textsubscript{P}, Co\textsubscript{N}, and Ni\textsubscript{N} (0.02-0.06 mg/mL), Y\textsubscript{2}O\textsubscript{3-P}, and Y\textsubscript{2}O\textsubscript{3-N} (0.05-0.12 mg/mL), CB\textsubscript{C}, and CB\textsubscript{P} (0.01-1 mg/mL). Before using, suspensions were put in an ultrasonic bath for 20 min and were shaken by vortex. Cells at 80 % of confluence were treated with material suspensions (500 \textmu L per well) for viability tests and incubated for 24 h under a humidified 5 % CO\textsubscript{2} atmosphere at 37 °C. For proliferation tests, cells at 40 % of confluence were treated with material suspensions (500 \textmu L
per well) and incubated for 24 and 48 h. Cultures treated with the same volume of the medium alone (DMEM supplemented with 5% (v/v) FBS) were used as controls.

**MTS assay**

The MTS assay which applies (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) salt was used to determine the cytotoxicity of materials according to the manufacturer’s instruction (Cell Titer 96® AQueous Non-Radioactive Cell Proliferation, Promega, USA). Briefly, after incubation time cells were rinsed with sterile phosphate buffered saline (PBS, pH 7.4). 300 μL of culture medium (DMEM supplemented with 5% (v/v) FBS) and 60 μL of MTS reagent were added to each well in the absence of light and cells were incubated for 2 h in humidified environment at 37 °C under 5% CO₂. Subsequently supernatants were transferred to 96-well plates and the optical density was quantitatively measured on a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at 490 nm.

**Neutral red uptake assay**

The neutral red assay was also used to investigate the cytotoxicity of the materials. Briefly, following exposure to materials, cells were rinsed with sterile PBS and incubated for 2 h with neutral red dye (100 μg/mL) dissolved in DMEM. Cells were then washed with PBS and fixed by formaldehyde 3% (v/v) followed by extracting the dye from the cells using EtOH/AcCOOH, 50%/1% (v/v) under gentle shaking for 15 min at RT. 100 μL of the extracted dye was transferred to 96-well plates and the absorbance was measured at 540 nm.

**LDH assay**

Lactate dehydrogenase (LDH) assay is a rapid measurement of the release of LDH enzyme from cells with a damaged membrane. LDH measurements were carried out using the CytoTox-ONE™ Homogeneous Membrane Integrity assay (Promega, USA) according to the manufacturer’s instructions. Briefly, the supernatants were collected after incubation of the cells with materials. 50 μL of each supernatant was transferred to a 96-well plate followed by the addition of 50 μL of CytoTox-ONE™ reagent to each well and incubated for 10 min at RT in the absence of light. 50 μL of stop solution was eventually added to rapidly stop the
continued generation of fluorescent product, and finally the optical density was measured at 490 nm.

In all the colorimetric assays performed in this study, each experimental condition had a corresponding “blank” containing the same concentration of material suspension but without presence of cells. Prior to analysing the spectrophotometer data, the absorbance of blank, which reflects the absorbance related to the materials, was subtracted from the absorbance of the experimental condition to measure only the color change due to the cell response to the material.

3.4.5 Actin and DNA staining

Swiss 3T3 mouse fibroblasts seeded in 35 mm x 10 mm cell culture dishes at 10,000 cell/cm² were grown to reach 40 % or 80 % of confluence and then were exposed to the highest concentrations of materials used in the cytotoxicity assays (i.e. 0.06 mg/mL for Ni, Co, and Mo, and 0.12 mg/mL for Y₂O₃). The concentration of 0.1 mg/mL was chosen for CB because the highest concentration (i.e. 1 mg/mL) covered the surface of the cells and resulted in totally preventing the cell observation under optical microscope. After 24 h (for 80 % of confluence) or 48 h (for 40 % of confluence) exposure to materials, cells were washed with PBS and fixed with 3 % (w/v) paraformaldehyde in PBS for 10 min at RT and then permeabilized for 5 min with 0.5 % (v/v) Triton X100 in PBS. After rinsing twice with PBS, non-specific binding sites were blocked by incubation with 1 % (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37 °C under a humidified 5 % CO₂ atmosphere. Following rinsing with PBS, F-actin and DNA were stained using rhodamine-phalloidin (Invitrogen, USA) diluted 1/200 with 0.1 % (w/v) BSA in PBS and 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) diluted 1/1,000 with PBS respectively. Dishes were incubated for 30 min at 37 °C under a humidified 5 % CO₂ atmosphere. After washing with PBS, dish walls were removed. Dishes were mounted in PBS/glycerol (50:50 (v/v)) for microscopy observation using an Eclipse TE2000-S microscope equipped with a 60 x objective and a Retiga 1300R camera (Nikon).
3.4.6 TUNEL apoptosis assay

To label apoptotic cells, Click-iT® TUNEL Alexa Fluor® Imaging assay (TUNEL, Invitrogen, Canada) was employed according to the manufacturer’s instructions. Briefly, the cells were seeded in a 96-well plate at 5 000 cell/cm² and grown to reach 80 % of confluence. Subsequently, they were exposed to the highest concentrations of materials (i.e. 0.06 mg/mL for Ni, Co, and Mo in all forms, 0.12 mg/mL for Y₂O₃) and incubated for 24 h at 37 °C under a humidified 5 % CO₂ atmosphere. Cells were then fixed using 3 % (w/v) paraformaldehyde in PBS for 15 min at RT and permeabilized using 0.25 % (v/v) Triton X100 in PBS for 20 min at RT and eventually washed twice with milliQ water. DNase I solution which creates strand breaks in the DNA was added to the positive control cells to provide a positive TUNEL reaction with 30 min incubation at RT. The TdT reaction buffer was added to each well and plate was incubated for 10 min at RT. Thereafter, cells were incubated with the TdT reaction cocktail for 60 min at 37 °C. Afterwards, cells were washed twice with 3 % (w/v) BSA in PBS for 2 min following by immediate addition of the Click-iT® reaction cocktail to the wells and incubation for 30 min at RT while being protected from light. Each well was rinsed with 3 % (w/v) BSA in PBS for 5 min. To obtain DNA staining, Hoechst 33342 solution was added and cells were incubated for 15 min at RT in the absence of light. Finally each well was washed twice with PBS and samples were taken for microscopy observation using an Eclipse TE2 000-S microscope equipped with a 20x objective and a Retiga 1300R camera (Nikon).

3.4.7 Determination of catalyst ion release

Catalysts were suspended in DMEM supplemented with 5 % (v/v) FBS at their highest concentrations used in this study (i.e. 0.06 mg/mL for Co, Ni, Mo and 0.12 mg/mL for Y₂O₃) and kept for 24 h at 37 °C inside the water bath to provide similar conditions to the incubation of cells with materials at 37 °C. The supernatant of the suspensions were transferred to another bottle and centrifuged at 1 000 rpm for 10 min. The new supernatant was further filtered using a 0.22 μm pore size nylon filter. After treating cells (80 % confluent for viability tests or 40 % confluent for proliferation tests) with the filtered supernatant for 24 or 48 h, MTS and neutral red assays were performed. Moreover, ICP-MS analysis was performed on the filtered supernatants to determine the dissolution of catalyst particles in the medium.
3.4.8 Statistical analysis

All statistical computations were performed with the Statistical Analysis System (SAS-9.2, Cary, NC, USA) using Tukey-Kramer multiple comparison test (ANOVA). Values were considered significantly different if p<0.05.

3.5 Results

3.5.1 Material characterization

Prior to cytotoxicity evaluation, each particle was analyzed for various physicochemical properties. Collected information on particle characteristics including particle shape and morphology (HRSEM), specific surface area (BET), particle size distribution (laser diffraction), composition and crystalline structure (XRD), purity (ICP-MS), and surface chemistry (XPS) is presented in Figure 3.1 and Table 3.1.

Size, morphology, and specific surface area of the particles

The particle size of commercial catalysts varied in micrometric range as shown in Table 3.1. Commercial CB powder consisted of primary particles with less than 50 nm size. However, they formed micrometric-sized aggregates. Depending on the material, the RFITP process could affect the primary particle size and/or morphology of the particles (Figure 3.1). Indeed, plasma treatment reduced the primary particle size of the metallic catalysts (Ni, Co, Mo, and Y$_2$O$_3$) from micrometric range to nanometric range. Plasma treatment also changed the morphology of all catalysts to spherical form except Ni which had spherical morphology even before plasma treatment. Surface area is one of the most important parameters in toxicological studies particularly nanotoxicology [Duffin et al., 2002]. Results of surface area measurement (BET) are summarized in Table 3.1. Along with the reduction in the particle size of the catalysts, their surface area increased significantly after plasma treatment. For instance, surface area of Ni$_p$ particles increased about 4 times in comparison with Ni$_c$ particles. Mo$_p$ and Y$_2$O$_3$$_p$ demonstrated surface areas respectively more than 20 and 2 times bigger than Mo$_c$ and Y$_2$O$_3$$_c$. Plasma treatment changed neither the primary particle size nor the surface area of the CB powder significantly. To summarize, plasma treatment (a) reduced the particle
size into nanometric ranges (b) changed the morphology into spherical one and (c) increased
the surface area of metallic catalyst particles.

Figure 3.1  Morphology of the studied catalysts and carbon black (CB) particles. HRSEM
images of different magnifications showing the morphology of the commercial
(\text{c}), plasma treated (\text{p}) and commercial nano-sized (\text{n}) particles.
Table 3.1 Properties of particles used in the present study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Particle size (mm)</th>
<th>Purity (wt %)</th>
<th>Crystalline structure</th>
<th>Surface area (m²/g)</th>
<th>Surface chemistry (wt %)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni_c₁</td>
<td>&lt; 1 *</td>
<td>99.5 *</td>
<td>Cubic Ni</td>
<td>2</td>
<td>Ni:69.9 O:24.9 C:5.1</td>
<td>Cerac (CANADA)</td>
</tr>
<tr>
<td>Co_c₁</td>
<td>&lt; 2 *</td>
<td>99.8 *</td>
<td>Cubic &amp; hexagonal Co</td>
<td>1</td>
<td>Co:75.3 O:18.7 C:4.5</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>Y₂O₃_c₁</td>
<td>&lt; 4⁴ (&lt;325 mesh) *</td>
<td>99.9 *</td>
<td>Cubic Y₂O₃</td>
<td>11</td>
<td>Y:67.6 O:25 C:6.5</td>
<td>Hermann C.Starck (THAILAND)</td>
</tr>
<tr>
<td>Mo_c₁</td>
<td>1-2 *</td>
<td>99.9 *</td>
<td>Cubic Mo</td>
<td>0.47</td>
<td>Mo:60.6 O:29.8 C:6.7</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>CB_c₁</td>
<td>0.039 *</td>
<td>&gt;96</td>
<td>Partially-graphitized</td>
<td>53</td>
<td>C:97.4 O:1.9</td>
<td>Columbian (USA)</td>
</tr>
<tr>
<td>Ni_p²</td>
<td>80% &lt;0.1</td>
<td>&gt;90</td>
<td>Cubic Ni/ Rhombohedral Ni₃S₂</td>
<td>8</td>
<td>Ni:62.5 O:24.9 C:12.4</td>
<td>-</td>
</tr>
<tr>
<td>Y₂O₃_p²</td>
<td>Mostly &lt;0.1</td>
<td>&gt;92</td>
<td>Cubic Y₂O₃</td>
<td>22</td>
<td>Y:64.6 O:26.2 C:5.4</td>
<td>-</td>
</tr>
<tr>
<td>Mo_p²</td>
<td>Mostly &lt;0.5⁴</td>
<td>&gt;95</td>
<td>Cubic Mo/ Monoclinic MoO₂</td>
<td>11</td>
<td>Mo:60.3 O:27.8 C:6.5 Y:4.7</td>
<td>-</td>
</tr>
<tr>
<td>CB_p²</td>
<td>Mostly &lt;0.1</td>
<td>&gt;92</td>
<td>Partially-graphitized</td>
<td>44</td>
<td>C:98.2 O:1.7</td>
<td>-</td>
</tr>
<tr>
<td>Ni_N³</td>
<td>&lt;0.1 *</td>
<td>99.9 *</td>
<td>Cubic Ni / Cubic NiO</td>
<td>6</td>
<td>Ni:78.2 O:17.8 C:3.9</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>Co_N³</td>
<td>0.005-0.015 *</td>
<td>99.9 *</td>
<td>Hexagonal Co/ Cubic Co₃O₄</td>
<td>42</td>
<td>Co:42.9 O:47.73 C:8.67</td>
<td>Alfa Aesar (USA)</td>
</tr>
<tr>
<td>Y₂O₃_N³</td>
<td>&lt;0.0 5 *</td>
<td>99.9 *</td>
<td>Cubic Y₂O₃</td>
<td>36</td>
<td>Y:64.6 O:23.7 C:4.5 CI:5.8 Mg:12</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
</tbody>
</table>

* Supplier data, ¹ Wide and non-uniform size distribution, ² Commercial, ³ Plasma treated, ⁴ Commercial nano-sized
Composition, crystalline structure, and purity of the particles

Crystalline structure and chemical composition of the particles in all forms (i.e. commercial, plasma treated and nano-sized) were determined using XRD analysis. Results showed cubic crystalline structure for Ni\textsubscript{c} while Ni\textsubscript{N} was composed of both cubic Ni and NiO. After plasma treatment, the composition of Ni changed to a mixture of cubic Ni along with crystalline nickel sulfide (Ni\textsubscript{3}S\textsubscript{2}). This is an interesting observation because there was no trace of sulphur in the Ni\textsubscript{c}. The reason for formation of Ni\textsubscript{3}S\textsubscript{2} with no external sulphur source can be mainly due to the evaporation of small amounts of sulphur present in the graphite insert which is a part of the RFITP synthesis set-up used for SWCNT production to maintain the favorable temperature profile along the reactor [Kim et al., 2007a].

XRD results showed that both cubic and hexagonal crystalline structures were found for Co\textsubscript{c} while Co\textsubscript{N} particles were shown to consist of hexagonal Co in the core and cubic Co\textsubscript{3}O\textsubscript{4} on the surface. These results were in accordance with the information provided by the supplier. Y\textsubscript{2}O\textsubscript{3} powders in all three forms (i.e. Y\textsubscript{2}O\textsubscript{3}.c, Y\textsubscript{2}O\textsubscript{3}.p, and Y\textsubscript{2}O\textsubscript{3}.n) illustrated the cubic crystalline structure. Mo\textsubscript{c} particles showed cubic structure while cubic Mo and monoclinic MoO\textsubscript{2} were found in Mo\textsubscript{p}. Semi-graphitic crystalline structure was observed for both CB\textsubscript{c} and CB\textsubscript{p} particles. It seemed that the structure of CB stays unaffected after plasma treatment. To summarize, it seems that the most important impact of plasma treatment was on Ni particles in which Ni\textsubscript{3}S\textsubscript{2} was formed.

Materials were analyzed for quantification of the elemental metallic impurities by ICP-MS. Although the purity of the commercial particles was provided by the suppliers, they were analyzed once again to ensure that there is no contamination enabling to induce possible cytotoxic effects. The results confirmed the purity provided by suppliers as shown in Table 3.1. ICP-MS results revealed an acceptable degree of chemical purity for the plasma treated particles with no metallic impurity other than the main element. Results of carbon elemental analysis confirmed the high purity of CB after plasma treatment.
Surface chemistry of the particles

Particle surface chemistry is a very important component of nano-sized materials. As the particle size reduces, the proportion of atoms found at the surface increases relative to the proportion inside the volume resulting in more reactive groups on the surface of the particle. Small surface modifications on metallic nanoparticles may significantly influence materials cytotoxicity [Studer et al., 2010]. XPS analysis was performed on the sterilized particles to analyse the surface composition of the particles that will be in contact with cells. Results revealed that in addition to the element of the particle, carbon (C) and oxygen (O) are the main components of the outermost surface of all the particles. The presence of carbon on the surface of the particles could be due to the hydrocarbon contaminations adsorbed from environment. Traces of impurities (<1 wt %) on the surface were neglected. XPS spectrum of Mo_c and Mo_p clearly indicated the presence of C and high quantity of O and Mo atoms. Two forms of oxide (i.e. MoO_2 and MoO_3) were present on the surface of both Mo_c and Mo_p along with low content of pure Mo metal. Moreover, the presence of low content of Y atoms in the Mo_p sample was detected. High resolution XPS data indicated that these Y atoms are individual and not chemically bound to Mo atoms. Therefore, these results suggest that the Y atoms belong to the contamination in the reactor.

XPS spectra of Ni_c, Ni_N and Ni_p indicated the presence of C, O and Ni elements on the surface of these particles as well. The oxidation state of different Ni particles was in the form of NiO. In addition, Ni metal and Ni (OH)_2 were also present on the surface of all Ni particles. Contrary to XRD results obtained from the bulk material, sulphur (S) was not observed on the surface of Ni_p by means of XPS analysis. This can be due to the fact that Ni_2S_3 does not exist in thin layers (< 10 nm) of Ni_p surface or simply it is not detectible by XPS. Inability of XPS for carbide detection on the surface of several catalysts has been already reported [Rummeli et al., 2011]. Both Co_c and Co_N had C and O atoms in addition to Co. The higher oxygen content of Co_N compared to Co_c is due to a layer of oxide on the surface of these particles as indicated by the supplier. The oxidation state of both particles on the surface was in the form of Co_3O_4. XPS results of Y_2O_3 particles confirmed the presence of yttrium oxide on the surface of Y_2O_3_c, Y_2O_3_N and Y_2O_3_p. C was also detected on the surface of these particles. XPS results of Y_2O_3_N particles also revealed low amount (< 2 wt %) of MgCl_2 (magnesium
chloride). XPS analysis on CB_c, CB_p indicated C as a main element on the surface. However, a trace of O and S were observed in their XPS spectra as well.

3.5.2 Cytotoxicity of commercial particles

To determine the influence of commercial materials on cell viability, neutral red uptake assay was performed on Swiss 3T3 fibroblasts (at 80 % of confluence) exposed to commercial particles (i.e. Ni_c, Co_c, Y_2O_3_c, Mo_c and CB_c) for 24 h (Figure 3.2). For Co_c, a dose-dependent reduction in the cell viability was observed. Co_c affected the cell viability even at concentrations as low as 0.001 mg/mL significantly in comparison to control cells (P<0.05). When Swiss 3T3 fibroblasts were exposed to Ni_c, Mo_c, and Y_2O_3_c, no significant decrease in cell viability was observed at all concentrations tested as shown by neutral red assay. In the cultures treated with CB_c, reduction in the cell viability was observed for the highest concentration tested in the assay (i.e. 1 mg/mL). It should be mentioned here that CB makes a very thick and heavy solution at this concentration which completely covers the surface of the cells. Therefore, the reduction in number of viable cells in this experimental condition probably takes place because of the physical coverage of cells by CB_c.

To further confirm any cytotoxicity of commercial materials, cell proliferation was analyzed using MTS assay on Swiss 3T3 fibroblasts (at 40 % of confluence) exposed to raising concentrations of commercial particles for 24 and 48 h (Figure 3.3). Among all the commercial metallic catalysts, Co_c was the only catalyst that drastically affected the cell viability in a dose-dependent manner. Therefore, as expected, the most severe decrease in cell proliferation was also caused by Co_c. For example, the lowest concentration of Co_c caused more than 50 % reduction in the cell proliferation in comparison to the control after 24 and 48 h. In higher concentrations, the cytotoxic effect of Co_c was even more drastic. Ni_c, Mo_c and Y_2O_3_c both had also negative impacts on the cell proliferation after 24 and 48 h. Although 0.1 mg/mL of CB_c reduced the cell proliferation about 20 % after 24 h, cell growth continued between 24 and 48 h.
Figure 3.2 Viability of cells incubated with commercial particles for 24 h.
The effect of commercial material concentration on the viability of Swiss 3T3 cells after 24 h incubation was studied using neutral red assay. Absorbance was recorded at 540 nm and normalised to untreated control cells. Results are expressed as means +SD of two independent experiments performed in triplicate for Co_c and Mo_c. For Ni_c, Y_2O_3_c and CB_c results are expressed as means +SD of a single experiment performed in triplicate as a representative of at least two independent experiments. (*) shows statistically significant difference compared to untreated control cells (p<0.05).
Figure 3.3 Proliferation of cells incubated with commercial particles for 24 and 48 h. The effect of commercial material concentration on the proliferation of Swiss 3T3 cells seeded was studied after 24 and 48 h using MTS assay. Absorbance was recorded at 490 nm and normalised to untreated control cells of 24 h. Results are expressed as means +SD of two independent experiments performed in triplicate. (*) shows statistically significant difference compared to untreated control cells at 24h (p<0.05) and (n) shows statistically significant difference between 24 and 48 h (p<0.05).

3.5.3 Cytotoxicity of plasma treated particles

To study the possible effects of synthesis process on the properties of the produced nanoparticles and their corresponding cytotoxicity, Swiss 3T3 fibroblasts were incubated with suspensions of plasma treated catalysts and CB in medium for 24 h. As mentioned before, plasma treated particles were synthesized from commercial particles. Because Co_C showed the greatest toxic effect on Swiss 3T3 fibroblasts within 24 h even at very low concentrations, we decided to remove this catalyst from the feedstock materials and not to use it any further for safety reasons. The effects of plasma treated materials on the Swiss 3T3 fibroblasts at 80 % of confluence were studied by neutral red, MTS (Figure 3.4 a) and LDH assays (Figure 3.4 b). Throughout all the experiments, neutral red and MTS assays revealed comparable results for all tested materials. Ni_p generated a very toxic effect on Swiss 3T3 fibroblasts regardless of the material concentration. LDH assay confirmed the results obtained by neutral red and MTS assays as very high amount of LDH was detected in the cultures exposed to Ni_p.
particles. In contrast, concentrations of Mo_p up to 0.06 mg/mL showed no significant effect on the viability of Swiss 3T3 fibroblasts in comparison to the control cells as demonstrated by neutral red, MTS and LDH assays. Viability assays also indicated that exposure to Y_2O_3_p for 24 h slightly decreased the viability of cells in comparison to the control cells (p<0.05). This result was confirmed by the low but still significant LDH activity detected in the supernatant of Swiss 3T3 fibroblasts incubated with Y_2O_3_p for 24 h in comparison to control cells (Figure 3.4 b). Neutral red and MTS assays showed that only the highest concentration (1 mg/mL) of CB_p induced significant effect on the viability of Swiss 3T3 fibroblasts which could be a result of the CB blanket that covered the surface of the cells as discussed before. Nevertheless, the increase in the LDH activity observed using the highest concentration of CB_p was not statistically significant.

The effect of plasma treated particles on the proliferation of Swiss 3T3 cells was also determined (Figure 3.5). As expected, among plasma treated metallic particles Ni_p made the most drastic dose-dependent reduction of the cell proliferation after 24 and 48 h. The cell growth was drastically decreased after 24 h incubation with Ni_p in comparison to the control cells. This effect was even more pronounced after 48 h confirming the high toxicity of Ni_p. Less severe cytotoxic action was observed in the cells exposed to Y_2O_3_p in comparison to the control cells. Mo_p which was shown to have no effect on the viability of sub-confluent Swiss 3T3 fibroblasts (Figure 3.4) caused a slight dose-dependent proliferation reduction. The effect was significant only at the highest concentration. In addition, the cells were able to grow significantly between 24 and 48 h in the presence of Mo_p at 0.04 and 0.06 mg/mL. CB_p showed no significant effect on the proliferation of Swiss 3T3 fibroblasts after 24 and 48 h.
Figure 3.4 Viability of cells incubated with plasma treated particles for 24 h by (a) neutral red and MTS assay and (b) LDH leakage assay.

The effect of plasma treated material concentration on the viability of Swiss 3T3 cells after 24 h incubation was studied using neutral red and MTS and LDH assays. Absorbance was recorded at 540 nm for neutral red and 490 nm for MTS and LDH assays and normalised to untreated control cells. Results are expressed as means ±SD of two independent experiments performed in triplicate except for the results of MTS assay for Mo₃, Y₂O₃, CB, and neutral red assay for CB which are expressed as means ±SD of a single experiment performed in triplicate as a representative of at least two independent experiments. (*) shows statistically significant difference compared to untreated control cells (p<0.05).
Figure 3.5  Proliferation of cells incubated with plasma treated particles for 24 and 48 h. The effect of plasma treated material concentration on the proliferation of Swiss 3T3 cells after 24 and 48 h incubation was studied using MTS assay. Absorbance was recorded at 490 nm and normalised to untreated control cells at 24 h. Results are expressed as means ±SD of two independent experiments performed in triplicate. (*) shows statistically significant difference compared to untreated control cells at 24h (p<0.05) and (n) shows statistically significant difference between 24 and 48 h (p<0.05).

3.5.4 Cytotoxicity of commercial nano-sized particles

The response of Swiss 3T3 fibroblasts to commercial nano-sized Ni, Co, and Y₂O₃ particles was investigated using (a) neutral red and MTS assays and (b) LDH leakage assay to compare them with the results obtained from the plasma treated catalysts (Figure 3.6). Results of both neutral red and MTS assays showed that Co₆N induced a slight dose-dependent toxicity in Swiss 3T3 fibroblasts. The fibroblasts exposed to 0.02 mg/mL of Co₆N have similar viability to the control cells (p>0.05) while exposure to 0.06 mg/mL of Co₆N significantly reduced the cell viability in comparison to the control cells (p<0.05). Moreover, LDH assay demonstrated that the cell membrane was not significantly damaged by Co₆N except for the cells exposed to the highest concentration tested (i.e. 0.06 mg/mL). Ni₆N particles imposed a slight reduction in the cell viability only at high concentrations and LDH assay revealed some cell membrane
damage only with the highest concentration (0.06 mg/mL) of Ni\textsubscript{N} particles. Neutral red and MTS assays did not reveal any significant reduction in lysosomal staining and metabolic activity respectively in the cells after being exposed to Y\textsubscript{2}O\textsubscript{3}-N. However, LDH assay showed significant amounts of the enzyme in the supernatant of the cells treated with Y\textsubscript{2}O\textsubscript{3}-N.

Figure 3.6 Viability of cells incubated with commercial nano-sized particles by (a) neutral red and MTS assay and (b) LDH leakage assay. The effect of commercial nano-sized material concentration on the viability of Swiss 3T3 cells after 24 h incubation was studied using neutral red and MTS and LDH assays. Absorbance was recorded at 540 nm for neutral red and 490 nm for MTS and LDH assays and normalized to untreated control cells. Results are expressed as means +SD of two independent experiments performed in triplicate except for the MTS assay for Co\textsubscript{N} and Y\textsubscript{2}O\textsubscript{3}-N, and neutral red assay for Y\textsubscript{2}O\textsubscript{3}-N which are expressed as means +SD of a single experiment performed in triplicate as a representative of at least two independent experiments. (*) shows statistically significant difference compared to untreated control cells (p<0.05).
The results of proliferation assessment performed on Swiss 3T3 fibroblasts treated with nano-sized metallic particles for 24 and 48 h is depicted in Figure 3.7. Co₉N made a dose-dependent proliferation reduction after 24 and 48 h. In agreement with viability assays results, Ni₉N had also a negative impact on the growth of Swiss 3T3 fibroblasts after 24 and 48 h. Y₂O₃₉N affected the proliferation of Swiss 3T3 fibroblasts after 24 and 48 h.

![Figure 3.7 Proliferation of cells incubated with commercial nano-sized particles for 24 and 48 h. The effect of commercial nano-sized particles material concentration on the proliferation of Swiss 3T3 cells seeded was studied using MTS assay after 24 and 48 h incubation. Absorbance was recorded at 490 nm and normalized to untreated control cells at 24h. Results are expressed as means +SD of two independent experiments performed in triplicate. (*) shows statistically significant difference compared to untreated control cells at 24h (p<0.05).](image)

### 3.5.5 Staining of actin and DNA

To analyse any changes in the actin cytoskeleton and visualize condensed chromatin due to apoptosis, F-actin and DNA were labeled in Swiss 3T3 fibroblasts incubated with the highest concentrations of materials for 48 h (Figure 3.8). The observations confirmed the results obtained with mitochondrial enzymatic assays. Control cells as well as cells treated with different forms of Mo, Y₂O₃ and CB, were spread and actin stress fibers were formed. In cultures treated with Co₉C and Ni₉P few cells remained attached without organization of the actin cytoskeleton. DAPI staining of those cells also revealed condensed chromatin indicating apoptosis.
Figure 3.8 DNA and F-actin of cells after 48 h incubation with particles.

DNA and actin cytoskeleton of Swiss 3T3 cells were studied by DAPI and rhodamine-phalloidin staining respectively after 48 h incubation with the highest concentration of each tested catalyst in this study (i.e. 0.06 mg/mL of Co, Ni, Mo, and 0.12 mg/mL of Y$_2$O$_3$). Concentration of the CB was selected to be 0.1 mg/mL to be able to observe cells. Cells were visualized under fluorescent microscopy (magnification 60 x). Results are representative of two independent experiments performed in duplicate.
3.5.6 TUNEL apoptosis assay

TUNEL assay was performed to determine whether the Swiss 3T3 fibroblasts exposed to various particles for 24 h underwent apoptosis (Figure 3.9). The nucleus of the apoptotic cells (positive control) treated with DNase I that generates strand breaks in the DNA were stained red, while in normal and healthy cells the nuclei were stained blue. The highest concentration of catalysts (i.e. 0.06 mg/mL for Co, Ni, and Mo, 0.12 mg/mL for Y$_2$O$_3$) were used for this assay. Results revealed that the cells treated with particles other than Co$_c$ and Ni$_p$, were mostly alive and there was no sign of apoptotic cells present in such cultures. Indeed, most of the Swiss 3T3 fibroblasts incubated with Co$_c$ were positively stained by TUNEL and some apoptotic cells were also clearly identified in the presence of Ni$_p$.

![Figure 3.9](image_url)

Figure 3.9  Cell death through apoptosis after incubation with catalyst particles. TUNEL apoptosis assay was performed to distinguish Swiss 3T3 cells undergoing apoptosis after 24 h incubation with the highest material concentration used in this study (i.e. 0.06 mg/mL of Co, Ni, and Mo and 0.12 mg/mL of Y$_2$O$_3$). Cells were observed under fluorescent microscopy (magnification 20 x). In positive control cells DNase I solution has created DNA strand breaks and nuclei are stained red. In negative control nuclei of cells are stained blue by Hoechst 33342 solution.
3.5.7 Cytotoxicity of released ions

To be able to distinguish the direct cytotoxic effect of the particles from their indirect effect due to the release of ions arose from the dissolution of the particles, the quantity of ions released from the particles was determined using ICP-MS. Among all the catalysts, significant dissolution occurred for Co particles. As shown in Table 3.2, about 35% of Co C was dissolved in culture medium. Dissolution of Mo in culture medium was also significant. For Ni and Y$_2$O$_3$, dissolved amounts of less than 1% indicated a low solubility in DMEM supplemented with 5% FBS under the applied conditions for these particles. To verify whether dissolution of particles plays a role in the cytotoxicity, cells were grown until 80% confluence and then were treated with filtered supernatant of suspensions at the highest concentration tested for each catalyst particle (i.e. 0.06 mg/mL for Co, Ni, and Mo, 0.12 mg/mL for Y$_2$O$_3$). Results of neutral red and MTS assays indicated that none of the supernatants had significant effect on the viability of Swiss 3T3 cells. To further verify whether dissolved particles have cytotoxic action, their influence on the proliferation of cells was determined after 24 and 48 h (Figure 3.10). Although slight but significant negative effect on cell proliferation at 24 h was observed with supernatant of Co C and Ni P, the cells were able to proliferate between 24 and 48 h in all experimental conditions. These results suggested that the cytotoxicity of the catalysts is mostly related to the direct contact between cells and materials and less due to the release of ions in culture medium.

Table 3.2 Concentration of metallic elements in supernatant filtered from the suspension with the highest concentration of each catalyst.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (mg/mL)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni C (0.06 mg/mL)</td>
<td>0.002136</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ni P (0.06 mg/mL)</td>
<td>0.00161</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ni N (0.06 mg/mL)</td>
<td>0.00121</td>
<td>0.00226</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Co C (0.06 mg/mL)</td>
<td>0.02136</td>
<td>0.00161</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Co N (0.06 mg/mL)</td>
<td>0.02033</td>
<td>0.00161</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y$_2$O$_3$ C (0.12 mg/mL)</td>
<td>0.00046</td>
<td>0.00045</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y$_2$O$_3$ P (0.12 mg/mL)</td>
<td>0.00046</td>
<td>0.00045</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y$_2$O$_3$ N (0.12 mg/mL)</td>
<td>0.00092</td>
<td>0.00092</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mo C (0.06 mg/mL)</td>
<td>0.00939</td>
<td>0.00939</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mo P (0.06 mg/mL)</td>
<td>0.01919</td>
<td>0.01919</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(Supensions containing the highest concentration of catalyst particles in cell culture medium were centrifuged at 5'000 rpm for 5 min and then filtered through a 0.22 μm pore-size nylon filter. The amount of corresponding element was measured in the filtered supernatants using ICP-MS analysis.)
Figure 3.10  Effect of the particles dissolved in the cell culture medium on the proliferation of cells after 24 and 48 h incubation. Proliferation of cells incubated with the filtered supernatants of suspensions with the highest concentration of each catalyst particle was studied by MTS assay. Absorbance was recorded at 490 nm and normalized to untreated control cells at 24 h. Results are expressed as means ±SD of two independent experiments performed in triplicate. (*) shows statistically significant difference compared to untreated control cells at 24 h (P<0.05), (+) shows statistically significant difference compared to untreated control cells at 48 h (P<0.05) and (n) shows statistically significant difference between 24 and 48h (p<0.05).
3.6 Discussion

The study of the response of Swiss 3T3 cells to commercial, plasma treated and commercial nano-sized particles provided the opportunity to understand how different properties of materials can affect their cytotoxicity because cells were exposed to materials with similar composition but different properties.

Because some limitations and inaccuracies have been reported for assays used for studying engineered nanomaterial cytotoxicity, it is recommended to use more than one assay for precise evaluation of nanoparticle toxicity [Monteiro-Riviere et al., 2009]. Therefore, in this study, three cytotoxicity assays based on enzymatic activity or organelle staining were chosen to study the effect of particles on the cell viability. Reduction in the metabolic activity of the cells is an early indication of cellular damage and can be measured by MTS assay [Baltrop et al., 1991] which is a convenient indirect method to measure number of viable cells [Cory et al., 1991]. Moreover, MTS assay is proven to be one of the most accurate assays in determining the cytotoxicity of nanomaterials [Monteiro-Riviere et al., 2009]. Neutral red assay is based on the ability of viable cells to incorporate neutral red dye in their lysosomes [Borenfreund and Puerner, 1984; Repetto et al., 2008]. The procedure is very sensitive and easily quantifiable and shows the number of viable cells in the culture indirectly. LDH assay shows membrane integrity damage which is a consequent of a serious injury and is applied to estimate cell death by LDH enzyme leakage. This assay measures the number of dead or seriously damaged cells in the culture [Mitchell et al., 1981].

3.6.1 Commercial particles

Neutral red assay results revealed that among all the commercial catalyst particles examined in this study only exposure of Swiss 3T3 fibroblasts to Co_C (< 2 μm) for 24 h resulted in an evident dose-dependent reduction of their viability. In contrast to our findings, Machado et al. (2011) have shown that Co particles with size distribution of 0.3 to 2 μm cause no reduction in the viability of immortalized human lung epithelial cells (A549) after 48 h at concentrations of 2.5-10 μg/mL assessed by MTS assay [Machado et al., 2011]. However, they showed that Co particles with size distribution of 0.01-1 μm significantly reduced the viability of A549 cells.
after 48 h [Machado et al., 2011] implying that among particles mainly composed of Co metal, particle size influences the cytotoxicity. The greatest cytotoxicity of Co_c was confirmed by proliferation assay at 24 and 48 h compared to other commercial catalysts.

During the proliferation assays, the number of cells in contact with the same concentration of materials was at first lower than that used during the viability tests and the incubation time was also elongated to 48 h. Therefore, it is reasonable to get more severe results in term of cytotoxicity with proliferation assays. Indeed, the viability of Swiss 3T3 fibroblasts at 80 % of confluence was not affected by Ni_c after 24 h, while it induced a decrease of the cell proliferation after 24 and 48 h. We also observed using optical microscopy that considerable amount of Ni_c particles seemed to be internalized by cells after 24 h (data not shown). It has been reported that the high quantity of particle uptake could be due to the chemical nature of the particle that may interact with proteins present in culture medium and be more readily taken up by cells [Oberdorster et al., 2005b; Ponti et al., 2009]. Pietruska et al. (2011) who exposed human lung epithelial cells (H460) to micron-sized Ni (~ 3 μm) with concentrations of 10-500 μM (i.e. 0.0005-0.02 mg/mL) for 24, 48, and 72 h reported that Ni particles were internalized by H460 cells and could be visualised within cytoplasmic vacuoles after 24 h. However, they found that Ni particles did not induce any cytotoxic effect as assessed by PicoGreen dsDNA kit [Pietruska et al., 2011]. It would be very interesting in a future work to confirm the ability of cells to internalize the nanoparticles in our experimental conditions using methods such as confocal microscopy with Z-stacking.

Different concentrations of Mo_c particles induced no effect on the viability of Swiss 3T3 fibroblasts at 80 % of confluence after 24 h in accordance with the findings of Sakai et al. (2002) who evaluated the effect of up to 0.6 mg/mL of micron-sized Mo (3-5 μm) on osteoblast-like cells (MG-63) by neutral red assay and found no significant effect after 6 days [Sakai et al., 2002]. However, it was shown in this study that when cells were in proliferative state, a dose-dependent reduction in the cell growth was observed in cells incubated with Mo_c for 24 and 48 h.

In line with the existing evidence that Y and Y_2O_3 are not considered cytotoxic [Andelman et al., 2010], it was shown that incubation with Y_2O_3_c influenced the morphology of cells in a
way that a lot of vacuoles were formed inside the cells. However, no significant reduction in
the cell viability after 24 h and only a moderate negative effect on the cell proliferation were
observed after 24 and 48 h incubation with Y$_2$O$_3$. It was also found that the cell viability was not affected by 0.01 and 0.1 mg/mL of CB$_3$, whereas incubation with 1 mg/mL of CB$_3$, induced about 50% reduction in the number of viable cells. Even though a slight decrease in proliferation was observed in the cells incubated with up to 0.1 mg/mL of CB$_3$, the cells continued to grow between 24 and 48 h indicating a low toxic effect of CB$_3$ on Swiss 3T3 fibroblasts. Yamawaki and Iwai (2006) studied human umbilical vein cells (HUVECs) treated with 1-100 µg/mL of CB for 24 h and found a dose-dependent cytotoxic morphological changes such as cytosolic vacuole formation, cell disorientation, and decreased density as well as increased LDH release and cell death in these cells [Yamawaki and Iwai, 2006]. Reisetter et al. (2011) reported cytotoxicity assessed by LDH assay and pyroptosis which is a proinflammatory form of cell death in RAW264.7 macrophages after exposure to 100 µg/mL of CB nanoparticles for 24 h [Reisetter et al., 2011]. In a recent study, internalization and cytotoxicity of two manufactured CB particles from Evonik/Degussa Co. (i.e. CB13 with primary particle size of 13 nm and CB21 with primary particle size of 21 nm) on MRC5 cells, a human fibroblast cell line, was determined. They showed that MRC5 cells internalized both CB particles similarly but mitochondrial enzymatic assay (WST-1) showed that 10 µg/cm$^2$ of CB13 induced about 70% loss of viability after 24 h, whereas CB21 had no effect on the viability of MRC5 cells [Belade et al., 2012].

**3.6.2 Plasma treated particles**

Thermal plasma processes produce nanoparticles because of the rapid temperature decrease and suppressed grain growth in such systems [Shigeta and Murphy, 2011]. The difference between the cytotoxic effects of the commercial and the plasma treated materials could be either due to the decrease of the particle size after passing through the plasma or because of the increase of the specific surface area. It can also be related to the change in the crystalline structure and composition of the particles that occurred because of the specifications of the synthesis set-up used to produce the plasma treated particles. For
instance, plasma treatment altered the cytotoxic effect of Ni particles. Ni\(_p\) showed a very strong cytotoxic effect for all the concentrations tested on Swiss 3T3 fibroblasts in comparison to Ni\(_c\).

The effect of plasma treatment is more moderate for Y\(_2\)O\(_3\). Y\(_2\)O\(_3\)-C and Y\(_2\)O\(_3\)-P had similar effects on cell morphology and proliferation at 48 h.

Surprisingly, it seems that plasma treatment improved the biological response to Mo catalysts because Mo\(_p\) particles affected the proliferation of Swiss 3T3 fibroblasts less than Mo\(_c\) particles. Moreover, as it was expected, because plasma treatment did not change the properties of CB, no strong difference was observed between the cytotoxic behaviors of CB\(_c\) and CB\(_p\).

### 3.6.3 Commercial nano-sized particles

Co\(_N\) particles at 0.02 mg/mL had less effect on the viability of cells than Co\(_c\) which is completely cytotoxic at the same dose. However, neutral red, MTS and LDH assay showed that incubation with 0.06 mg/mL of Co\(_N\) reduced the number of viable cells in the culture after 24 h.

Our results are in agreement with those of Peters et al. (2004) who reported a decrease in cellular viability after exposure to 0.05 mg/mL of nano-sized Co particles (50-200 nm) in human dermal microvascular endothelial cells (HDMEC) using MTS assay [Peters et al., 2004].

Incubation with Ni\(_N\) particles decreased the survival of Swiss 3T3 fibroblasts at 80 % of confluence, while Ni\(_c\) particles had no effect. This result is in accordance with the results of Pietruska et al. (2011) who exposed H460 cells to nano-sized Ni particles (<100 nm) with concentrations of 10-500 \(\mu\)M (i.e. 0.0005-0.02 mg/mL) for 24, 48 and 72 h and observed intermediate toxicity using PicoGreen dsDNA kit [Pietruska et al., 2011]. In another study, Ni nanoparticles have shown a moderate toxicity on human alveolar epithelial A549 and monocyte/macrophage cells determined with MTT assay after 24 h [Lanone et al., 2009].

Significant cytotoxic effect was observed in the cells incubated with Y\(_2\)O\(_3\)-N particles, only when LDH assay was applied while MTS and neutral red assays showed no effect on the cell
viability. However, in proliferation state, MTS assay revealed a moderate reduction in cellular growth after 24 and 48 h incubation with Y\textsubscript{2}O\textsubscript{3-N} particles. In agreement with our findings, Y\textsubscript{2}O\textsubscript{3} nanoparticles of 12 nm particle size have been reported to be relatively non-toxic to hippocampal nerve cells (HT22) and mouse macrophages (RAW164) after 20 h (MTT assay). In addition they are able to rescue HT22 cells from oxidative stress-induced cell death. Therefore, it is suggested that Y\textsubscript{2}O\textsubscript{3} nanoparticles can have antioxidant properties that promote cell survival under oxidative stress and have a potential to be used for therapeutic purposes [Schubert et al., 2006].

3.6.4 Released ions

Results of ICP-MS analysis on the supernatants of the catalysts suspensions showed that dissolution mainly took place for Co and Mo particles. Despite the high amount of dissolved Co\textsubscript{C} (35 % of the available Co), neutral red and MTS assays on sub-confluent cells showed no reduction in the viability after 24 h incubation with the supernatant, unlike the cytotoxicity results obtained with Co\textsubscript{C} particles. Proliferation assay showed that dissolved Co\textsubscript{C} caused a slight reduction in cellular growth after 24 h. Therefore, our results suggest that a direct contact of suspended material must be established to reduce the cell viability significantly. Ponti et al. (2009) have suggested a possible synergic effect of ions released in culture medium and/or the particles on the toxicity of nano-sized Co. They studied the cytotoxicity of nano-sized Co (20-500 nm) at the concentration range of 0.1-100 \( \mu \)M (i.e. 0.0059-5.9 \( \mu \)g/mL) compared to its soluble form (i.e. CoCl\textsubscript{2}) on Balb/3T3 mouse fibroblasts. They observed an increase in the percentage of Co\textsuperscript{2+} release from nano-sized Co in culture medium after 24h exposure measured by \( \gamma \)-counter reaching ~ 20 % for 100 \( \mu \)M. In their study, cytotoxicity evaluated by colony-forming efficiency (CFE) test showed dose-dependent responses obtained for nano-sized Co and Co\textsuperscript{2+} after 24h of exposure. Therefore, they could not conclude if the observed toxicity was resulted from nano-sized Co particles or the released ions because they observed an effect for both Co\textsuperscript{2+} and nano-sized Co [Ponti et al., 2009]. Issa et al. (2008) have studied the viability of human gingival fibroblasts (HGF) after 24h exposure to metal salts. They showed that more than 50 % loss of viability occurred in cells exposed to Co\textsuperscript{2+} at 0.04 mg/mL (800 \( \mu \)M) [Issa et al., 2008].
Similar to the results obtained with Mo particles, dissolved Mo (15%) in culture medium did not induce any significant cytotoxicity in cells. In agreement with our cytotoxicity results for Mo, Sakai et al. (2002) showed that Mo particles did not affect the viability of osteoblast-like cells after 6 days. However, they reported a slight toxicity by Mo suspension extracts containing dissolved ions suggesting that high local concentration of dissolved Mo ions in contact area between particles and cells induces a weak cytotoxic effect on MG-63 cells [Sakai et al., 2002].

Our results showed that the dissolution of different types of Ni particles was very low and for all Y$_2$O$_3$ particles the release of Y was measured to be less than 1%. In line with our results, Pietruska et al. (2011) have reported mobilization of only 1-3% of the available Ni in Ni nanoparticles (< 100 nm) and a minimal mobilization of Ni from micron-sized Ni particles (~3 μm) even after 72 h [Pietruska et al., 2011]. The viability of HGF exposed to 0.04 mg/mL (800 μM) of Ni$^{2+}$ was decreased about 40% after 24 h [Issa et al., 2008]. Lu et al. (2009) showed that treatment of mouse L-929 fibroblast cell line with 200 mM Ni$^{2+}$ (prepared using NiCl$_2$.6H$_2$O) for 24, 48 and 72 h caused a time-dependent increase in cell cytotoxicity relative to the control culture and incubation with 500 μmol/L Ni$^{2+}$ decreased the proliferation of L-929 fibroblasts more than 40% as assessed by MTT assay [Lu et al., 2009].

Rudolf and Cervinka (2010) investigated the effect of Ni (nickel sulfate) on human primary skin fibroblasts using WST-1 assay and found a concentration-dependent cytotoxicity of the material with a 50% loss of viability through apoptosis at 250 μM (0.01 mg/mL) and 90% loss of viability at 1000 μM (0.05 mg/mL) [Rudolf and Cervinka, 2010].

Thus, toxicity of the metallic particles beyond released ion toxicity could be mainly due to the settlement of the particles at the bottom of the culture dish and their direct contact with the adherent cell monolayer that leads to a possible pro-oxidative stress on the cell membrane components and/or to a mechanical damage of the cells by the particle aggregates [Horev-Azaria et al., 2011].
3.6.5 Correlation between particle properties and cytotoxicity

Particle size

Our findings show that if a material is not toxic to cells in micrometric size range, reduction in its particle size (without any changes in composition or other properties) will not necessarily make it toxic as proven by comparing the cytotoxicity of Mo_C and Mo_P after 24 h. The viability of Swiss 3T3 fibroblasts incubated with Mo_C or Mo_P was similar although Mo_P has smaller particles than Mo_C. Moreover, Mo_P seemed less cytotoxic than Mo_C in terms of cell proliferation. It was found that Y_2O_3_C with micrometric particle size had similar effect on the proliferation of Swiss 3T3 fibroblasts compared to Y_2O_3_P and Y_2O_3_N with nanometric particle sizes.

However, for some particles reduction in particle size can result in an increase in the cytotoxicity as observed for Ni_C and Ni_P particles. Nevertheless, regarding to the distinct cytotoxicity observed for Ni_P and Ni_N which are both nanoparticles of Ni with similar nanometric particle sizes, it is difficult to merely attribute the cytotoxicity of Ni_P to its particle size reduction. Taking into account all cell toxicity results, we cannot conclude that particle size reduction to nanometric ranges necessarily yields in an increased cytotoxicity for all materials. It seems that the primary particle size cannot be the only responsible factor in the cytotoxicity of fine materials [Murdock et al., 2008]. Most of the nanomaterials do not necessarily retain their nanometric size once they are in solution and they usually make agglomerations with micrometric size especially in a biological medium [Mackay et al., 2006; Sager et al., 2007]. Besides, dispersion of nanomaterials in liquid rarely leads to distribution of those materials at the primary particle size. For instance, sonication slightly decreases agglomeration of the particles in the solution while it has no significant effect on the particle surface charge. Therefore, particles would make agglomerates again after a period of time [Murdock et al., 2008]. Poorly dispersed suspensions undergo substantial agglomeration during cellular experiments. This issue has a major influence on the effective dose of the particles delivered to the cells. Consequently, a large part of the cytotoxicity differences may be caused by variations in the actual particle dose delivered to the cells throughout the experiments. Therefore, it is important to follow appropriate protocols for nanoparticle...
dispersion [Taurozzi et al., 2011]. It has been recently shown that 5% FBS is considered as one of the best dispersing agents for stable nanoparticle dispersion preparation in the cell culture media including DMEM [Ji et al., 2010]. This procedure was used for material dispersion in our cytotoxicity experiments.

**Particle surface area**

The issues regarding the most appropriate metric of dose for toxicological studies are still unsolved. Surface area is the parameter usually proposed to represent at best the toxicity of nanoparticles [MacNee and Donaldson, 2003]. On the other hand, several reports indicated that mass is the most appropriate metric [Pauluhn, 2009]. It was therefore decided to consider both mass and surface area concentrations. The corresponding surface area for each tested mass concentration was measured using BET analysis. According to the results of viability assays for different commercial particles, it was found that Co_C at the dose of 0.2 cm²/mL (equal to 0.02 mg/mL) is drastically toxic. However, when cells were exposed to Ni_C and Mo_C with the same surface area (equal to 0.01 mg/mL and 0.05 mg/mL for Ni_C and Mo_C respectively), no significant reduction of the cell viability was observed. Furthermore, comparing the results of the viability assays for different particles of the same element, it was also found that cytotoxicity cannot solely be attributed to the surface area. The surface area of Mo_P is more than 20 times bigger than Mo_C particles but they still did not cause significant death of sub-confluent Swiss 3T3 fibroblasts. Among Ni particles, the surface area of Ni_P and Ni_N particles have increased about 4 and 3 times compared to Ni_C particles. However, the strong cytotoxicity of Ni_P particles, proved by three cytotoxicity assays, cannot be attributed only to the bigger surface area of these particles because Ni_N particles that have comparable surface area to Ni_P, induced much less cytotoxic effects in Swiss 3T3 fibroblasts. It has been discussed previously in the literatures that a more reactive surface chemistry, will have a more profound effect when surface area is increased [Oberdorster et al., 2007]. The reactive surface area which depends on a combination of different properties such as size, chemistry and dispersibility may therefore constitute a more accurate measure of particle toxicity [Nel et al., 2009].
Particle composition and chemistry

Co₃C₃ was shown to be the most harmful particle to Swiss 3T3 fibroblasts among all the examined catalyst particles in this study while Co₃N₃ particles induced cell death only at high concentrations. The reason why no strong toxicity was observed with Co₃N₃ in comparison to Co₃C₃, despite its smaller particle size and bigger surface area, may be due to the fact that Co₃N₃ particles used in this study are composed of a Co core surrounded by a Co₃O₄ shell as indicated by the supplier. Mostardi et al. (2010) exposed human synovial fibroblasts to 0.004 or 0.04 g of two CoCr particles (noted by A or B). While similarly internalised by the fibroblasts, the CoCr-A with less elemental Co present on the surface was significantly less (P < 0.05) deadly to the fibroblasts than the CoCr-B, in which XPS analysis had shown more considerable elemental Co on the surface suggesting that lower surface cobalt content being presented to the cells may significantly reduce cytotoxicity [Mostardi et al., 2010].

Ni₃P₃ particles induced a strong toxicity in Swiss 3T3 fibroblasts. One important difference between Ni₃P₃ and other forms of Ni examined in this study is the presence of crystalline Ni₃S₃ formed in Ni₃P₃ particles as shown by XRD analysis which is a highly toxic material. Nickel sulfide compounds contain a class of inorganic compounds whose potent carcinogenic activity is highly dependent on their crystalline structure [Abbracchio et al., 1982]. Crystalline Ni₃S₂ is reported to be rapidly phagocyted by Chinese hamster ovary and Syrian hamster embryo cells causing strong toxicity after 24 h [Costa and Mollenhauer, 1980].

For future investigations, it would be very interesting to evaluate the impact of these materials on human bronchial epithelial cells and also human macrophages involved in inflammatory responses.

3.7 Conclusion

Our study draws attention of nanomaterial industry particularly CNT producers to consider the toxicity of feedstock materials. Comparing the cytotoxicity of a group of popular metallic catalysts used for CNT production, Co particles were found to be the most cytotoxic catalyst among Co, Ni, Mo, and Y₂O₃. RFTTP process can affect the physicochemical properties and cytotoxicity of the catalysts used to produce CNTs. Ni catalysts became strongly cytotoxic
after RFITP treatment which might be due to nickel sulfide formation. To summarise, the experimental data suggest that:

- Criteria like size and surface area of the particles cannot fully explain the observed cytotoxicity of the catalysts.

- Particle composition that can be modified during the RFITP process may be the most reliable property to justify the catalyst cytotoxicity.

- Release of ions from the catalysts cannot strongly impair the cell proliferation and survival.

To conclude, it is important to be aware of the influence of the synthesis process on the physico-chemical properties of materials and their toxicity in nanomaterial industries including CNT related processes.

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Declaration of Interest

This study was supported financially by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Research Chair of Canada (Laboratory of Cell-Biomaterials Biohybrid Systems). The authors declare that they have no competing interests. The authors are responsible for the content and writing of the paper.
CHAPTER 4 Synthesis of single-walled carbon nanotubes using induction thermal plasma technology with different catalysts: thermodynamic and experimental studies

Avant-propos

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Titre français: Synthèse de nanotubes de carbone mono-parois par une technologie de plasma thermique inductif avec différents catalyseurs: étude expérimentale et analyse thermodynamique

Contribution to the document:

This article contributes to the thesis by developing a hypothesis that the induction thermal plasma process has the capacity to produce high quality SWCNTs using non-toxic catalysts. Moreover, the quality of the produced SWCNT material is affected by the type and amount of
catalysts. A ternary mixture of Ni, Co, and \( \text{Y}_2\text{O}_3 \) has been applied to produce high quality SWCNTs by RF induction thermal plasma process. However, as shown in the previous chapter, these catalyst particles revealed distinct cellular effects and commercial Co particles showed high cytotoxicity. Therefore, we aimed to replace Co by other less harmful catalyst particles in the feedstock and evaluate the ability of the system to produce high quality SWCNTs with the new catalyst mixtures. In order to reach this objective, different ratios of the metallic catalysts were applied in the synthesis process. Moreover, performing thermodynamic calculations of the tested systems and comparing them with experimental results allowed us to better understand the role of each catalyst in the production of SWCNTs and propose solutions to be able to apply non-toxic catalysts for high quality SWCNT synthesis. All of the thermodynamic equilibrium calculations were performed based on the minimization of Gibbs free energy using FACTSAGE code v. 6.2. The calculations were performed in a closed system at a constant pressure of 67 kPa and a temperature range of 500 to 5000 K. The gas phase is assumed ideal while the solution phase is considered to be real and the non-ideality term is included in the Gibbs free energy minimization calculation through the RKM (Redlich-Kister-Muggianu) polynomial for the excess term.

The experimental works as well as the thermodynamic studies and drafting of the manuscript were performed by Yasaman Alinejad and Ali Shahverdi under the supervision of Prof. Gervais Soucy and Prof. Nathalie Faucheux.

4.1 Résumé

Les effets du type et de la quantité de trois mélanges de catalyseurs (i.e., Ni-\( \text{Y}_2\text{O}_3 \), Ni-Co-\( \text{Y}_2\text{O}_3 \), et Ni-Mo-\( \text{Y}_2\text{O}_3 \)) sur la synthèse de nanotubes de carbone mono-parois (SWCNTs) par un procédé plasma thermique inductif ont été examinés afin d'évaluer leurs influences individuelles sur les SWCNTs produits. Les calculs thermodynamiques, en gaz et en particulier dans les phases de la solution liquide, ont également été réalisés afin de mieux comprendre les effets des catalyseurs sur la production de SWCNTs. La caractérisation des SWCNTs synthétisés qui a été réalisée par différentes techniques dont la spectroscopie Raman, la microscopie électronique à balayage à haute résolution (HRSEM) et les analyses
thermogravimétriques (TGA) a clairement montré que la meilleure qualité de SWCNTs est obtenue en utilisant le mélange de catalyseur de Ni-Co-Y2O3.

4.2 Abstract
The effects of the type and quantity of three catalyst mixtures (i.e. Ni-Y2O3, Ni-Co-Y2O3, and Ni-Mo-Y2O3) on single-walled carbon nanotubes (SWCNTs) synthesis by induction thermal plasma process have been examined in order to evaluate their individual influences on SWCNT production. Thermodynamic calculations, in gas and particularly in liquid solution phases, have also been performed to better understand the effects of the catalysts on the production of SWCNTs. Characterization of the synthesized SWCNTs by different techniques including Raman spectroscopy, high resolution scanning electron microscopy (HRSEM) imaging and thermogravimetric analysis (TGA) clearly indicated that the best quality of SWCNTs was achieved using Ni-Co-Y2O3 catalyst mixture in the feedstock.

4.3 Introduction
Due to the very exceptional thermal, mechanical and electrical properties [Baddour and Briens, 2005] of single-walled carbon nanotubes (SWCNT) discovered by Iijima in 1993 [Iijima and Ichihashi, 1993], much attention has been attracted to this novel nanostructured material. It has a broad range of applications in different fields such as sensors [Cao and Rogers, 2009; Genest et al., 2012], polymers [Breuer and Sundararaj, 2004; Ci et al., 2008], microelectric devices [LeMieux et al., 2009], and biomedical engineering [Fadel et al., 2010; Zanello et al., 2006]. Different methods have been developed for the synthesis of SWCNT including arc discharge [Iijima and Ichihashi, 1993], laser ablation [Guo et al., 1995], chemical vapor deposition (CVD) [Dai et al., 1996], and induction thermal plasma [Kim et al., 2007a]. These methods can be divided into two main categories depending on their catalytic or catalyst free synthesis. In catalytic synthesis methods, the presence of catalysts, mainly transition metals, in the feedstock or on the support plate is inevitable [Moisala et al., 2003]. In laser ablation and arc discharge methods high temperature heating of the target, made from graphite-catalyst mixture plate, followed by a rapid cooling rate process of the generated vapor results in the formation of high quality SWCNT. However, in the induction thermal plasma
method (ITP), the formation of SWCNT is based on the direct evaporation of feedstock materials containing carbon black (CB) and a mixture of catalysts in the high temperature plasma plume. For CVD methods, hydrocarbon decomposition takes place over the supported catalyst particles which indeed act as active sites for the nucleation of SWCNT [Dai et al., 1996]. Since different phenomena occur in various synthesis methods, it is somehow impossible to adopt a unique mechanism for the SWCNT formation. Among all suggested mechanisms so far, the nucleation and growth of SWCNT based on carbon-catalyst interaction [Celnik et al., 2008] is more suited for the gas phase methods. Indeed, in the gas phase, SWCNT formation includes several steps (a) decomposition of catalysts and carbon source, (b) nucleation and growth of catalyst nanoparticles, (c) carbon diffusion or solubilization into the metal particles and (d) precipitation and solidification in the form of SWCNT. In this mechanism, diffusion and solubilization of carbon atoms into the catalyst liquid droplets is a key factor. The rate of dissolution is mainly dependent on the type of catalyst, temperature, and the presence of other solutes. For example, it has been shown that small amount of sulfur can reduce the surface tension of liquid metal which favors the higher solubility of carbon [Flint, 1965]. It has been shown experimentally that the solubility of carbon in different catalysts varies notably. For example, the solubility of carbon is high in Mo and Fe, while it is very low in Cu [Moisala et al., 2003]. Therefore, it is expected that changes in the type of catalyst can distinctly affect the formation of SWCNT. Although the efficiency of different catalysts in production of SWCNT with the other methods of synthesis has been intensively studied, no systematic experimental study has been conducted on the effect of the type and quantity of catalysts on the SWCNT synthesis by ITP.

In the present work, since the solubility of carbon and the gas phase composition of plasma are expected to affect the SWCNT synthesis, a complete thermodynamic study on a wide temperature range (500-5 000 K) was carried out in order to fully understand the effect of the type and quantity of catalyst on the gas-phase during the synthesis of SWCNT by ITP process. The operating temperature window was limited to $T < 5 000$ K since at higher window temperature of plasma the system is noticeably far from the equilibrium state. It is expected that at lower temperatures considered in this work, thermodynamic studies will help to better understand the effect of different catalysts on the synthesis of SWCNT.
Following the thermodynamic study, the effect of three different catalyst mixtures on the SWCNT synthesis by ITP was experimentally studied. Along with Yttrium oxide ($Y_2O_3$), Ni, Co, and Mo have been selected due to their high efficiency in production of SWCNT using catalytic synthesis methods [Moisala et al., 2003]. Moreover, since there is controversial data about the toxicity of metallic catalysts in the literature [Machado et al., 2011; Sakai et al., 2002; Tessier and Pascal, 2006], it is important to find less harmful catalysts that are capable of producing high quality SWCNT.

4.4 Thermodynamic calculations

Theoretical thermodynamic study was carried out using FACTSAGE 6.2 software on the different carbon-catalyst mixture systems summarized in Table 4.1. To study the solution phase, particularly in liquid state the FSstel database was selected for the calculations. For the gas phase, the FACT53 database was considered without taking into account the inert gases (Ar, He). Moreover, the presence of sulfur in the CB was not neglected since it can alter the quality of SWCNT [Alinejad et al., 2010]. For these series of calculations, two groups of mixtures were considered and their results were compared. In Group 1 (i.e. MIX 1, 2 and 3) three different mixtures of catalysts were considered with a constant total amount of catalysts (i.e., 5.2 wt %). In fact, the Co content present in MIX 1 was replaced by the same amount of Ni in MIX 2 and by the same amount of Mo in MIX 3 so that the total metallic catalyst content remained constant. Group 2 which includes MIX 4, MIX 5 and MIX 6, contains mixtures with different ratios of Ni-$Y_2O_3$ and Ni-Mo-$Y_2O_3$.

4.5 Experimental procedure and set-up

SWCNT were synthesized at large-scale using ITP system (feed stock injection rate of 2 g/min), as shown in Figure 4.1. Detailed information about the experimental set-up can be found in a previous paper [Kim et al., 2007a]. The physical properties of the feedstock materials are summarized in Table 4.2. The same mixture compositions used for thermodynamic calculations (Table 4.1) were used in the experimental studies.
Table 4.1. Feedstock material content in weight percent (wt %) used for the synthesis of SWCNTs.

<table>
<thead>
<tr>
<th>MIX no.</th>
<th>Ni</th>
<th>Co</th>
<th>Mo</th>
<th>Y$_2$O$_3$</th>
<th>CB</th>
<th>S*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>2.6</td>
<td>2.6</td>
<td>0</td>
<td>7.5</td>
<td>87.3</td>
<td>2</td>
</tr>
<tr>
<td>2*</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>7.5</td>
<td>87.3</td>
<td>2</td>
</tr>
<tr>
<td>3*</td>
<td>2.6</td>
<td>0</td>
<td>0</td>
<td>7.5</td>
<td>87.3</td>
<td>2</td>
</tr>
<tr>
<td>4*</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>85</td>
<td>2</td>
</tr>
<tr>
<td>5*</td>
<td>5.2</td>
<td>0</td>
<td>0.5</td>
<td>7.5</td>
<td>86.8</td>
<td>2</td>
</tr>
<tr>
<td>6*</td>
<td>1.3</td>
<td>0</td>
<td>3.9</td>
<td>7.5</td>
<td>87.3</td>
<td>2</td>
</tr>
</tbody>
</table>

* wt % of sulfur in CB, * Group 1, * Group 2

Figure 4.1 Schematic diagram of an ITP system designed for large-scale synthesis of high quality SWCNTs.

The synthesized SWCNT samples were characterized by means of Raman spectroscopy, X-ray diffraction (XRD, PANALYTICAL-X'Pret Pro MPD), thermogravimetric analysis (TGA, SETSYS 2400) and high resolution scanning electron microscopy (HRSEM, S4700, Hitachi).
Raman spectroscopy was performed with Ar laser ($\lambda_{\text{exc}} = 514$ nm). TG was performed according to the protocol provided in [Shahverdi and Soucy, 2012].

Table 4.2  Properties of the materials used for the production of SWCNTs.

<table>
<thead>
<tr>
<th>Material</th>
<th>Size</th>
<th>Purity (%)</th>
<th>$T_{\text{midRun}}$</th>
<th>$T_{\text{expected}}$</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>2µm</td>
<td>99.9</td>
<td>1455</td>
<td>2913</td>
<td>Cerac (CANADA)</td>
</tr>
<tr>
<td>Co</td>
<td>2µm</td>
<td>99.9</td>
<td>1495</td>
<td>2927</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>Mo</td>
<td>2µm</td>
<td>99.9</td>
<td>2623</td>
<td>4639</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>CB</td>
<td>45nm</td>
<td>98</td>
<td>N/A</td>
<td>3642</td>
<td>Grade: M280, Cabot Inc.(USA)</td>
</tr>
<tr>
<td>$Y_2O_3$</td>
<td>5µm</td>
<td>99.9</td>
<td>2425</td>
<td>4300</td>
<td>Hermann C.Starck (THAILAND)</td>
</tr>
</tbody>
</table>

4.6 Results and discussion

4.6.1 Thermodynamic study

Thermodynamic calculations were performed on different SWCNT feedstock mixtures summarized in Table 4.1. The results of thermodynamic calculations in gas phase and liquid solution phase on Group 1 are depicted in Figure 4.2.
The comparison of the total gas phase of each mixture revealed that the total gas phase was poorly affected by the type of metallic catalysts since it ended at almost the same temperature with the same pattern for all the mixtures. Nonetheless, slight differences were observed at the temperature where the total gas phase was terminated for the three mixtures. For example, a more extended gas phase at lower temperature was observed for the MIX 3, MIX 1, and MIX 2 respectively. Details of gaseous species are illustrated under the gas phase (total) curve.
(Figure 2, red curve). The concentration and evolution pattern of the most important precursors of SWCNT (i.e. C_i, i= 1 to 5) seemed not to be affected by the change in the catalyst mixtures. The exothermic reaction of Y-C along with other reactions such as Y-S, Y-O and Ni-S can be considered as a key phenomenon to improve the yield of SWCNT synthesis process since the released heat increases the temperature locally and enhances the supersaturation of carbon in liquid solution phase [Kim et al., 2009b]. The heat released due to Y-C and Y-S reactions will be higher as their concentration in gas phase are higher compared to other reactions, as seen in Figure 4.2 (a), (c) and (e). Figure 4.2 (b), (d) and (f) show the liquid solution phase formed during the high temperature plasma process of feedstock materials in Group 1. The graphs show that Y did not participate in liquid solution in contrast with the metallic catalysts (i.e., Ni, Co and Mo). The liquid solution was formed abruptly for all mixtures as seen in Figure 4.2. However, for MIX 3, (Figure 4.2 f), Ni content gradually increased up to a point (around $T=2800$ K) where it became the main solvent, while Mo started to diminish at $2500$ K due to solidification. The whole liquid solution phase solidified at a temperature lower than $1500$ K. The presence of liquid solution phase containing metals in such a wide range of temperature compared to the pure liquid metals is due to the solubilization of carbon which lowers the solidification temperature of metals [Gorbunov et al., 2002; Krivoruchko and Zaikovskii, 1998]. The liquid solution phases depicted in Figure 4.2 showed also that along with the presence of liquid metals as solvent, and carbon as a solute, the other species including O, S and NiS exist in all liquid solutions. Compared to other species, a high degree of solubilization for carbon in liquid metals favored the formation of SWCNT. Interestingly, Mo compared to other catalysts can markedly elongate the existence range of liquid solution phase about $1000$ K. The calculated liquid solution phase of MIX 1 and MIX 2 are depicted in Figure 4.3. The Mole-Temperature graphs of S and O for both mixtures were quite the same indicating that their solubility was the same in Ni and Co. However, three main differences between the liquid solution phase of MIX 1 and MIX 2 were observed (Figure 4.3). The first difference was the solubility pattern of C which started at a higher content for MIX 1 and ended at the same content as MIX 2. This observation firstly suggests that the solubility of C was enhanced by Co in the binary system of Ni-Co compared to unary Ni system. Moreover, taking into account the concentration gradient of C over
temperature as a driving force for nucleation of SWCNT [Celnik et al., 2008; Wen et al., 2008], the binary Ni-Co system, having a bigger $\Delta C/\Delta T$ in the same range of temperature than unary Ni system, eases the nucleation of SWCNT. The second difference was the range of temperature in which the liquid solution phase exists. As shown in Figure 4.3, this temperature range seemed to be slightly wider for MIX 1 than MIX 2. According to the very high temperature gradient and cooling rate along the induction thermal plasma reactor [Kim et al., 2009b], the wider temperature range in which the liquid solution phase exists can increase the time of SWCNT growth leading to a higher quality final product. The third difference is that unlike Ni, Co did not react with S in liquid solution phase which consequently allowed the active catalysts sites to uptake more carbon atoms.

Figure 4.4 and Figure 4.5 show thermodynamic calculations for another mixture of Ni (MIX 4) and two other mixtures of Mo (MIX 5 and MIX 6). Figure 4.4 shows the gas phase and liquid solution phase of MIX 4 in which the amount of Ni and $Y_2O_3$ was changed. By reducing the amount of $Y_2O_3$ in the feedstock mixture, the oxygen content was also reduced. The gas phase composition of the feedstock mixture was consequently affected by this change in the oxygen content as observed in the thermodynamic calculation results (Figure 4.4 a). On the other hand, the liquid solution phase was more affected by the change in the Ni content. High content of Ni in the gas phase makes the formation of NiS thermodynamically more favorable which in turn may partially compensate the diminution in the heat released by the exothermic reaction of $Y-C \rightarrow YC_2$. The shortage of O in the reaction system results in an increase in sulfur cluster ($S_2$) followed by the formation of another cluster ($S_3$) in the lower temperature regions ($T< 3000$ K) (Figure 4.4 a). The formation of the clusters of sulfur at temperatures where liquid solution phase exists can reduce the effectiveness of the process since these clusters can compete with carbon precursors in being solubilized into the liquid solution and occupy it and form the soluble NiS. This can be a reason why the amount of S and NiS in the liquid solution phase has been remarkably increased in Figure 4.4 b.
Figure 4.3  Superimposed liquid solution phase of MIX 1 (dashed-line) and MIX 2 (solid-line).

Figure 4.4  MIX 4 thermodynamic equilibrium compositions in (a) gas phase and (b) liquid solution phase.

The total gas phase and also carbon precursors (C₁-C₅) seemed to be independent from the amount of metal catalysts and follow the same pattern, as shown in Figure 4.5. The total heat of dominant exothermic reactions, mentioned above, was poorly affected since the gas phase composition did not significantly change between MIX 5, MIX 6, and MIX 3 as shown in Figure 4.2 c. The main changes were detectable from the liquid solution phases (Figure 4.5 b and d). By adding small amount of Mo (i.e., 0.5 wt %) the liquid solution phase temperature window and the solubility of solute species were changed compared to MIX 2. By increasing the amount of Mo, the solubility of C was changed compared to MIX 2 which is in total
agreement with the experimental observations where the solubility of carbon is very high in Mo [Moisala et al., 2003].

Figure 4.5 Thermodynamic equilibrium composition in gas phase (left side) and liquid solution phase (right side) of (a) MIX 5 and (b) MIX 6.

4.6.2 Experimental results

HRSEM images of SWCNT samples synthesized with different feedstock mixtures are depicted in Figure 4.6. All samples represent the existence of SWCNT bundles entwined with impurities including graphitic particles, catalysts and amorphous carbon. HRSEM images
demonstrated also the different morphology of SWCNT produced using different types and amounts of catalysts.

To compare the structural quality of SWCNT samples synthesized with different catalyst mixtures, Raman spectroscopy was performed and the results are summarized in Table 4.3. The intensity of tangential vibration mode of graphite at 1582 cm\(^{-1}\) (G-band), over the intensity of disordered sp\(^3\) carbon at 1350 cm\(^{-1}\) (D-band), is an informative value for the structural quality assessment of SWCNT [Dresselhaus et al., 2005]. A higher G/D ratio is an indicator for a better quality of SWCNT. The Raman analysis showed that the highest quality of the SWCNT was achieved using MIX 1 in which Co was present. In contrast, the lowest quality of SWCNT was obtained using MIX 6 in which Mo had the highest content. Sample obtained with MIX 2, possessed SWCNT with a lower quality than those produced by MIX 1. Therefore, Ni-Co binary mixture would be a better choice than Ni alone for production of high structural quality SWCNT. Furthermore, Co can be considered as a promoter for the synthesis of SWCNT. The results of Raman analysis for MIX 1 and MIX 2 are in good agreement with the thermodynamic calculations that predicted a superior potential of Ni-Co mixture in the synthesis of higher quality SWCNT. For MIX 4, SWCNT quality seemed similar to that obtained using MIX 2. This observation is in good agreement with the thermodynamic results. Although the increase and decrease in the Ni and Y\(_2\)O\(_3\) contents respectively, could affect the reaction system individually, their overall impact on the SWCNT synthesis seemed to be neutral.
Figure 4.6 HRSEM images of SWCNT samples synthesized with (a) MIX 1, (b) MIX 2, (c) MIX 3, (d) MIX 4, (e) MIX 5 and (f) MIX 6.

Table 4.3 Quality assessments of SWCNTs synthesized with different catalysts mixtures.

<table>
<thead>
<tr>
<th>MIX No.</th>
<th>MIX 1</th>
<th>MIX 2</th>
<th>MIX 3</th>
<th>MIX 4</th>
<th>MIX 5</th>
<th>MIX 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/D</td>
<td>15.2</td>
<td>13.6</td>
<td>4</td>
<td>14</td>
<td>4.5</td>
<td>3.8</td>
</tr>
</tbody>
</table>

To closely examine the crystalline structure of SWCNT synthesized with different catalyst mixtures in Group 1, XRD analysis was performed and the results are shown in Figure 4.7.
The XRD analysis revealed that unlike pure Ni and Co, pure Mo did not exist in the final SWCNT product. However, the carbide form of Mo (MoC) was found. This observation suggested the higher tendency of Mo to form carbide than Ni and Co catalysts in the present ITP operating conditions. It should be mentioned that the carbide formation can reduce the yield of the SWCNT synthesis by putting away the active catalysts from the SWCNT nucleation sites. Therefore, the formation of MoC in high quantity during the ITP process is unfavorable and has to be minimized.

To further investigate discrepancies observed between experimental results and thermodynamic calculation for Mo, the temperature profile of the reactor along the central axis was plotted regarding to the numerical simulation of the ITP synthesis system (Figure 4.8) [Kim et al., 2009b]. The eutectic temperature of Mo-C system ($T_{\text{eutectic}} = 2500$ K) [Hugosson et al., 2001] and Ni-C/Co-C ($T_{\text{eutectic}} = 1600$ K) [Kim et al., 2009b] are located at 0.6 and 0.75 m respectively from the torch exit (Figure 4.8). Two temperature zones are depicted in this graph: 3000-2000 K and 2000-1000 K. Since it has been experimentally proven that the optimal synthesis temperature lies 100-200 K below the $T_{\text{eutectic}}$ [Gorbunov et al., 2002; Kataura et al., 2000], the Zone 1 is expected to be a region in the ITP reactor which is more appropriate for the synthesis of SWCNT with Mo catalyst while the Zone 2 is suitable for using Ni and Co catalysts. As shown in Figure 4.8, the length of Zone 1 is only 13 cm in the reactor while the length of the Zone 2 is 30 cm. Therefore the nanotubes synthesized with Ni
and Co have at least 2.3 times greater residence time in their suitable temperature zone than those synthesized with Mo.

![Figure 4.8](image)

Figure 4.8  Axial profile of temperature along the centre line of the reactor. Black and white circle denote the eutectic temperature of Mo-C and Ni-C/Co-C systems, respectively.

Given that Co and Ni eutectic temperature is almost the same, this observation enables to explain why both Ni and Co produced SWCNTs with similar quality but higher than that synthesized using Mo. Therefore, to overcome the problem observed with Mo it is necessary to optimize the ITP operating conditions to obtain similar temperature zone for Mo with the same effective length as Ni and Co.

To characterize the thermogravimetric behavior of each SWCNT sample produced with the feedstock mixtures in Group 1, TG analysis was performed (Figure 4.9). All SWCNT samples demonstrated a multistep weight loss in the temperature range of 200-800 °C indicating the presence of different carbonaceous structures including SWCNT. The mass loss became zero at temperatures above 800 °C where the residual mass, attributed to the residual catalyst content, was calculated to be 15, 12 and 11 wt % for the SWCNT samples synthesized with MIX 1, MIX 2, and MIX 3, respectively. The mass gain at temperature around 200 °C was due
to the oxidation of metal catalysts in the samples which was then followed by a rapid mass loss at a temperature around 350 °C due to the fast reaction of amorphous carbon with oxygen [Shahverdi et al., 2012]. The temperature at which SWCNT samples start to lose weight (T onset) seemed to be identical. However, the multistep weight loss event (TG graph) was more identical for the samples synthesized with MIX 1 and MIX 3 than MIX 2. This observation suggested that the ternary mixture of catalysts with the identical catalyst oxide produced SWCNT samples with the same TG behavior while this behavior was slightly changed when the binary mixture was used.

![Figure 4.9 TG graphs of SWCNT samples synthesized using MIX 1 (dashed-dotted line), MIX 2 (dashed line) and MIX 3 (solid line).](image)

**4.7 Conclusion**

The effect of metallic catalysts and their quantity in the feedstock material on the synthesis of SWCNT were thermodynamically and experimentally studied. High quality SWCNT can be produced with a ternary catalyst mixture containing Ni, Co and Y₂O₃ or with a binary mixture of Ni and Y₂O₃. Thermodynamic calculations indicated that type and quantity of metal catalysts would affect more the liquid solution phase formed in the ITP reaction system during SWCNT production and less the gas phase, while the content of Y₂O₃ changed the gas phase with less impact on the liquid solution phase. Considering the eutectic temperature of the metallic catalyst-carbon system, the effective residence time for Ni/Co was found to be 2.3
times more than Mo in the ITP resulting in a higher quality Ni/Co-grown SWCNT. Thus, Mo potential for the synthesis of SWCNT can be improved through ITP process optimization by increasing the length of higher temperature zone ($T > 2000$ K).

**Acknowledgement**

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CHAPTER 5 Preosteoblasts behavior in contact with single-walled carbon nanotubes synthesized by radio frequency induction thermal plasma using various catalysts

Avant-propos

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Titre français: Comportement des préostéoblastes au contact de nanotubes de carbone mono-parois synthétisés par plasma thermique inductif en utilisant différents catalyseurs

Contribution to the document:

This article contributes to the thesis by developing a hypothesis that the SWCNTs produced from different catalyst mixtures may have diverse cytotoxicity. Here, the aim was to investigate the cytotoxicity of the SWCNT-containing final product of the RF induction thermal plasma process. Three SWCNT samples synthesized by applying different catalyst mixtures with the same total amounts but various components were chosen, characterized in detail, and evaluated in terms of their influences on the viability and proliferation of preosteoblasts. To properly evaluate the effects of these SWCNTs on the bone forming cells, we examined both exposure conditions (i.e. suspension or substrate) that are possible when
applying CNTs in bone applications and showed that the procedure applied for exposing cells to SWCNTs during cytotoxicity assessments can have an influence on the obtained results.

Yasaman Alinejad performed the synthesis and cytotoxicity evaluation experimental work, the statistical analyses, as well as, drafting the manuscript under the supervision of Prof. Nathalie Faucheux and Prof. Gervais Soucy.

5.1 Résumé
L'influence des nanotubes de carbone mono-parois (SWCNTs) produits par plasma thermique inductif avec trois mélanges de catalyseurs (Ni-Co-Y$_2$O$_3$, Ni-Y$_2$O$_3$, Ni-Mo-Y$_2$O$_3$) sur le comportement des préostéoblastes MC3T3-E1 murins a été évaluée. Après avoir analysé les propriétés des SWCNTs, les activités enzymatiques mitochondriales (MTS) et de lactate déshydrogénase (LDH) ainsi que l'incorporation du rouge neutre (NR) ont été mesurées pour évaluer la viabilité cellulaire. Afin de s'assurer que la cytotoxicité n'était pas simplement due à une perturbation mécanique, les SWCNTs ont été ajoutés sur les cellules attachées ou les cellules ont été ensemencées sur des plaques recouvertes de SWCNTs. Indépendamment des mélanges de catalyseurs utilisés pour leur production, les SWCNTs ajoutés sur les cellules attachées affectent considérablement la viabilité cellulaire de façon dose-dépendante. Toutefois, la viabilité des cellules ensemencées sur les SWCNTs en particulier sur ceux produits avec Ni-Co-Y$_2$O$_3$ n'a que légèrement diminué à 24 h. De plus, les cellules peuvent proliférer dans les 48 heures. D'autre part, les cellules ont été en mesure d'organiser leur cytosquelette d'actine et aucune cellule apoptotique n'a été détectée dans les cultures. Ainsi, sauf en cas de perturbation mécanique et rupture de la membrane cellulaire, les SWCNTs produits par plasma thermique ne semblent induire aucune cytotoxicité sévère sur les préostéoblastes MC3T3-E1 et sont donc considérés comme des CNTs prometteurs afin d'être plus particulièrement étudiés pour de futures applications en ingénierie tissulaire osseuse.

5.2 Abstract
The influence of single-walled carbon nanotubes (SWCNTs) produced by radio frequency induction thermal plasma with three catalyst mixtures (Ni-Co-Y$_2$O$_3$; Ni-Y$_2$O$_3$; Ni-Mo-Y$_2$O$_3$) was evaluated on the behavior of murine MC3T3-E1 preosteoblasts. After analyzing SWCNTs
properties, mitochondrial enzymatic (MTS) and lactate dehydrogenase (LDH) activities as well as neutral red (NR) uptake were measured to assess the cellular viability. To ascertain that the cytotoxicity was not merely due to the mechanical disturbance, either SWCNTs were added on the attached cells or cells were seeded on the SWCNT-covered plates. Regardless of the catalyst mixtures used for their production, SWCNTs added on the attached cells reduced cell viability drastically in a dose-dependent manner. However, the viability of cells seeded on SWCNTs even on those produced with Ni-Co-Y$_2$O$_3$ was slightly decreased at 24 h and besides, cells could proliferate within 48 h. Furthermore, cells were able to organize normal filamentous actin cytoskeleton and no apoptotic cells were detected in the cultures. Thus except mechanical disturbance and disruption of the cell membrane which were resulted from SWCNTs weight, thermal plasma grown SWCNTs seem to induce no severe cytotoxicity on MC3T3-E1 preosteoblasts and therefore are considered promising CNTs to be studied more deeply for future applications in bone tissue engineering.

5.3 Introduction

Carbon nanotubes (CNTs), also known as the most promising nanostructures of carbon, are expected to be produced in millions of tons worldwide in order to be used in numerous fields of application [Thayer, 2007]. For instance, they have a huge potential in biomedical applications as novel drug or gene delivery systems [Kateb et al., 2007; Wu et al., 2005]. Furthermore, their near infrared optical absorption properties make them suitable for the laser heating cancer therapy [Kam et al., 2005; Mahmood et al., 2012]. Interestingly although CNTs are known as the strongest materials on earth [Yu et al., 2000], they are much lighter than the typical materials used in bone implants such as steel and titanium [Zanello et al., 2006]. Therefore, CNTs can also be applied in bone tissue engineering in order to not only improve the mechanical properties of the biomaterials but also stimulate the repair process [Giannona et al., 2007; Saito et al., 2008; Zanello et al., 2006]. Bone matrix is mainly made of collagen and hydroxyapatite [Marquis et al., 2009]. The diameter range of SWCNTs lies between 0.7 - 1.5 nm which is very close to the size of the triple helix collagen fibrils. Therefore, SWCNTs have a good potential to be used as scaffolds for the nucleation and growth of hydroxyapatite crystals [Zhao et al., 2005]. However, biocompatibility and toxicity of CNTs should be
studied prior to use these promising nanostructures of carbon in biomaterials and bone tissue engineering. Several studies have been performed in recent years on the effect of CNTs on the proliferation and differentiation of bone cells. For example, chemical vapor deposition (CVD) grown multi-walled CNTs (MWCNTs) were shown to induce osteogenic differentiation of human adipose-derived mesenchymal stem cells [Li et al., 2012]. Another study demonstrated that the arc discharge or laser ablation grown SWCNTs were non-toxic to human osteoblasts in the same range as Ti6Al4V alloy (standard material used for implants) [Kalbacova et al., 2007]. In contrast, SWCNTs produced by HiPco process were reported to induce acute toxicity in MC3T3-E1 preosteoblasts [Tutak et al., 2009]. Nonetheless, an increase in the synthesis of extracellular matrix was also observed in the presence of these SWCNTs [Tutak et al., 2009]. Tutak et al. suggested that SWCNT-mediated cell destruction induced a release of endogenous factors that act to boost the activity of the surviving cells [Tutak et al., 2009].

Therefore, despite the huge amount of studies on CNTs toxicity in recent years [Helland et al., 2008; Johnston et al., 2010; Yang et al., 2009], the results are mostly contradictory because of the distinct properties of the CNTs used. CNTs can be single, double, or multi-walled depending on the number of layers present in their structure. Moreover, diameter size, length, morphology, quality and purity of CNTs can also vary distinctly [Paradise and Goswami, 2007]. The properties of CNTs are very dependent on the method applied for their synthesis [Hussain et al., 2009]. Indeed, CNTs can be produced by arc discharge [Iijima, 1991], laser ablation [Thess et al., 1996], CVD [Cassell et al., 1999], and radio frequency (RF) induction thermal plasma process [Kim et al., 2007a]. Moreover, various carbon sources such as carbon monoxide [Chiang et al., 2001] and carbon black (CB) [Alinejad et al., 2010], and a wide range of metallic catalysts including nickel (Ni), cobalt (Co), molybdenum (Mo), iron (Fe), etc. are usually used as a starting material in these processes [Ajayan et al., 1993; Dai et al., 1996; Journet et al., 1997; Kumar and Ando, 2010a; Lambert et al., 1994]. The as-produced product usually contains a mixture of amorphous carbon, metals, metal oxides and graphitic particles along with the CNTs [Wick et al., 2007]. The amount and chemistry of the impurities also depend on the process used for CNTs production [Journet et al., 2012]. It has been shown that each of these properties could play a role in the toxicity state of CNTs [Donaldson et al., 2006; Kostarelos, 2008; Shvedova et al., 2003; Wick et al., 2007].
In the present study, the cytotoxicity of SWCNTs produced by RF induction thermal plasma using three catalyst mixtures (Ni-Co-Y$_2$O$_3$; Ni-Y$_2$O$_3$; Ni-Mo-Y$_2$O$_3$) has been evaluated for the first time. The synthesized SWCNTs were characterized by means of different techniques such as high resolution scanning electron microscopy, thermogravimetric and X-ray diffraction analyses, and X-ray photoelectron and Raman spectroscopies. Consequently, the effects of SWCNTs (0-100 µg/mL, dose range used in numerous studies [Di Giorgio et al., 2011; Mu et al., 2009; Tian et al., 2006]) on the viability and proliferation of murine MC3T3-E1 preosteoblasts were evaluated using MTS, NR, and LDH assays. The MC3T3-E1 preosteoblasts were chosen as a model for osteoblasts, crucial cells for bone formation and repair, since they have typical osteoblastic developmental sequence [Bergeron et al., 2009; Takuwa et al., 1991].

In cytotoxicity evaluations, CNTs are studied either as dispersed in the biological environment or as embedded in a matrix [Hussain et al., 2009]. However, because CNTs are used in bone applications as dispersed in suspension [Mao et al., 2013; Mendes et al., 2010], embedded in a composite material [Van Der Zande et al., 2010] or as compacts [Li et al., 2012], we have investigated the behavior of bone cells in contact with SWCNTs in both conditions (in suspension or immobilized) at the same time. SWCNT suspensions were first prepared in cell culture medium and put on the cells attached to polystyrene (PS) culture plates. In the second strategy, culture plates were covered by SWCNT suspensions in ethanol followed by vaporization of ethanol. Cells were then seeded on the SWCNT-covered dishes as applied also by [Kalbacova et al., 2007]. Changes in the filamentous actin (F-actin) cytoskeleton and nuclei of the MC3T3-E1 preosteoblasts were tracked using rhodamine-phalloidin and DAPI staining respectively. TUNEL assay was also applied to identify the mechanism of cell death in cultures where viability was lost significantly.

5.4 Materials and methods

5.4.1 SWCNTs synthesis

SWCNT materials were synthesized by RF induction thermal plasma process as described previously [Kim et al., 2009b]. Briefly, the feedstock materials were injected through a water-
cooled probe at the center of a TEKNA PS-50 plasma torch (Tekna Plasma Systems, Inc, Sherbrooke, Qc, Canada) into the system which consists of a tubular reactor containing interchangeable graphite inserts for flexible control over temperature and a filtration system containing three metallic filters to collect the as-produced SWCNT materials. A mixture of argon and helium was used to provide the plasma and the synthesis processes were performed at 40 kW plate power and 67 kPa operating pressure.

Three SWCNT samples were synthesized as follows: The first SWCNT sample (SWCNT-1) was produced from a mixture of CB as the carbon source and Ni, Co and yttrium oxide (Y$_2$O$_3$) as the mixture of catalysts (CB-Ni-Co-Y$_2$O$_3$: 87.5-2.6-2.6-7.5 wt %). To produce the second SWCNT sample (SWCNT-2), Co was replaced by the same amount of Ni (CB-Ni-Y$_2$O$_3$: 87.5-5.2-7.5 wt %). In the third SWCNT sample (SWCNT-3), Co was replaced by the same amount of Mo (CB-Ni-Mo-Y$_2$O$_3$: 87.5-2.6-2.6-7.5 wt %). Indeed, the total amount of metallic catalyst content was kept constant. Properties of the materials used for SWCNT production are summarized in Table 5.1. The amorphous carbon content of all SWCNT samples was partially removed by thermal oxidation at 350 °C for 1 hour.

Table 5.1 Properties of the materials used for SWCNT production

<table>
<thead>
<tr>
<th>Material</th>
<th>Size</th>
<th>Purity (%)</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>2μm</td>
<td>99.9</td>
<td>Cerac (CANADA)</td>
</tr>
<tr>
<td>Co</td>
<td>2μm</td>
<td>99.9</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>Mo</td>
<td>2μm</td>
<td>99.9</td>
<td>Sigma-Aldrich (CANADA)</td>
</tr>
<tr>
<td>Y$_2$O$_3$</td>
<td>4μm</td>
<td>99.9</td>
<td>Hermann C.Starck (THAILAND)</td>
</tr>
<tr>
<td>CB</td>
<td>45nm</td>
<td>98</td>
<td>Grade: M280, Cabot Inc.(USA)</td>
</tr>
</tbody>
</table>

5.4.2 Material characterization

High resolution scanning electron microscopy (HRSEM, S4700; Hitachi, USA) was used to observe SWCNTs synthesized using the three catalyst mixtures. Moreover, the influence of different catalyst mixtures on the morphology of the produced SWCNTs was investigated.
Raman spectroscopy was performed on SWCNT samples in order to relatively and rapidly compare the quality of the SWCNT samples [Kim et al., 2007a]. Raman measurements were made using argon (514.5 nm) laser excitation.

To compare the purity of the SWCNTs produced by the three different catalyst mixtures, UV-Vis-NIR spectroscopy measurements were performed using a UV-Vis-NIR spectrophotometer (Carry 5000, Varian, CA, USA) on 10 mg of the sample dispersed completely in DMF using a sonication tip [Shahverdi et al., 2012]. The solution was diluted until an appropriate signal was obtained by the spectrometer.

Thermogravimetric analysis (TGA, TA instrument, TGA 2050, SETARAM, France) was used to distinguish various carbonaceous components of the SWCNT samples and the catalyst content of the samples as described previously [Shahverdi and Soucy, 2012]. For the TGA analysis, SWCNTs were oxidized in flowing air (40 sccm) using a temperature ramp of 10 °C/min from room temperature (RT) to 1000 °C.

Chemical composition of the SWCNT samples were analyzed by X-ray diffraction analyzer (XRD, PANALYTICAL-X’Pert ProMPD, Cu-Kα, λ=1.54Å).

X-ray photoelectron spectroscopy (XPS, AXIS ULTRA DLD spectrometer, monochromatic Al-Kα (hv = 1 486.69 eV) source at 225 W, photoelectron collection angle of 52.5 °) was performed on the samples to detect the elements present on the surface of the SWCNT samples. Energy resolution of the system (i.e., source and analyzer) was set to 0.3 eV. The elemental composition of the analyzed surface areas was obtained from survey spectra collected at pass energy of 160 eV.

5.4.3 Cell culture

Murine calvarial MC3T3-E1 preosteoblasts, subclone 14, (CRL-2594™, ATCC®, Manassas, VA, USA) were grown in minimum essential medium (MEM) alpha medium (αMEM, Gibco®, Grand Island, NY, USA) without ascorbic acid, supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Wisent, St-Bruno, Qc, Canada), 100 U/mL penicillin (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) at 37 °C under a humidified 5 % CO₂
atmosphere. Cell passage was performed using 0.25 % Trypsin-EDTA 1x (Invitrogen, Canada). Cells were used for the experiments between passages 3 and 13.

5.4.4 Cell viability and proliferation assays

SWCNTs seeded on MC3T3-E1 preosteoblasts attached to PS
SWCNTs were sterilized by heating at 180 °C for 3 h and their suspensions (0-100 µg/mL) were made in αMEM supplemented with 10 % (v/v) FBS. Before using, suspensions were put in an ultrasonic bath for 20 min and were shaken by vortex. The MC3T3-E1 preosteoblasts were seeded in 24-well plates (5 000 cells/ cm²) and allowed to attach and reach 40 % of confluence for proliferation and 80 % of confluence for viability assays. Cells were then treated with SWCNT suspensions (500 µL per well) and incubated for 24 and 48 h under a humidified 5 % CO₂ atmosphere at 37 °C. Cultures treated with the same volume of the medium alone (αMEM supplemented with 10 % (v/v) FBS) were used as controls. Each SWCNT concentration had a corresponding blank containing no cells.

MC3T3-E1 preosteoblasts seeded on SWCNT-covered PS
SWCNTs- (0-100 µg/mL) were suspended in pure ethanol and then the solutions were added to 24 well-plates. Plates were kept under the biological safety cabinet overnight to let the ethanol vaporize completely, creating a randomly distributed SWCNT film. Pure ethanol was also added to control wells. Cells were seeded on the SWCNT-covered well-plates (10 000 cells/cm² for viability and 5 000 cells/cm² for proliferation assays) and incubated for 24 and 48 h under a humidified 5 % CO₂ atmosphere at 37 °C.

Light microscopy imaging
Morphology of the MC3T3-E1 preosteoblasts treated with different concentrations of SWCNTs (0-100 µg/mL) was observed in all experimental conditions using a phase contrast Eclipse TE2000-S microscope with a 10x objective and a Retiga 1300R camera (Nikon, Mississauga, ON, Canada).
MTS assay
The viability of the MC3T3-E1 preosteoblasts and their proliferation were assessed using the mitochondrial enzyme activity assay according to the manufacturer's instruction (Cell Titer 96® AQuesous Non-Radioactive Cell Proliferation, Promega, Nepean, ON, Canada). Briefly, the MC3T3-E1 preosteoblasts were rinsed with sterile phosphate buffered saline (PBS, pH 7.4) after 24 h incubation with SWCNT suspensions with various concentrations at 37 °C and 5 % CO2. Then, 300 μL of culture medium (αMEM supplemented with 10 % (v/v) FBS) and 60 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) reagent were added to each well in the absence of light and the plate was incubated for 2 h in a humidified environment at 37 °C under 5 % CO2. Subsequently, supernatants were transferred to a 96-well plate and their optical density was measured at 490 nm by a microtiter plate reader (Bio-Tek, Winooski, VT, USA).
To evaluate cell proliferation, MTS assay was carried out on the MC3T3-E1 preosteoblasts, at 40 % of confluence, incubated with various concentrations of SWCNTs for 24 and 48 h.

NR assay
The NR assay was also used to investigate the viability of the MC3T3-E1 preosteoblasts. Briefly, following 24 h exposure to SWCNTs at different concentrations, the cells were rinsed once with sterile PBS (pH 7.4) and incubated for 2 h with NR dye (100 μg/mL) dissolved in αMEM. The cells were then washed with PBS and fixed by paraformaldehyde 3 % (v/v) followed by extracting the dye from the cells using EtOH/AcCOOH, 50 %/1 % (v/v) under gentle shaking for 15 min at room temperature (RT). 100 μL of the extracted dye was transferred to a 96-well plate and the absorbance was measured at 540 nm.

LDH assay
Lactate dehydrogenase (LDH) leakage from cells with a damaged membrane was measured using the CytoTox-ONE™ Homogeneous Membrane Integrity assay (Promega, USA) according to the manufacturer's instructions. Briefly, 50 μL of the collected supernatants of the MC3T3-E1 preosteoblasts incubated with SWCNTs for 24 h was transferred to a 96-well plate followed by the addition of 50 μL of CytoTox-ONE™ reagent to each well and
incubation for 10 min at RT in the absence of light. 50 µL of stop solution was added to rapidly stop the continued generation of product. The density of final solution was measured at 490 nm.

As stated before, in all colorimetric assays performed in this study, each experimental condition had a corresponding “blank” containing the same concentration of material suspension without cells. Prior to analyzing the spectrophotometry data, the absorbance of the blank, which reflects the absorbance related to the materials, was subtracted from the absorbance of the experimental condition to be able to measure only the color change that has occurred due to the cell response to the material.

5.4.5 Nuclei and cytoskeleton organization evaluated by DNA and F-actin staining

After 24 h exposure to SWCNTs, the MC3T3-E1 preosteoblasts were washed with PBS, fixed with 3 % (w/v) paraformaldehyde in PBS and then permeabilized with 0.5 % (v/v) Triton X100 in PBS for 5 min at RT. Non-specific binding sites were blocked by incubation of cells with 1 % (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37 °C. Filamentous actin (F-actin) and DNA were then stained respectively using rhodamine-phalloidin (Invitrogen, Burlington, ON, Canada) diluted 1/200 with 0.1 % (w/v) BSA in PBS and 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) diluted 1/1 000 with PBS. For microscopic observations, the walls of the dishes were removed and the remaining plates were mounted in PBS/glycerol (50:50 (v/v)). Cells were observed using an Eclipse TE2000-S microscope equipped with a 60x objective and a Retiga 1300R camera (Nikon).

5.4.6 TUNEL apoptosis assay

To label apoptotic cells, Click-iT® TUNEL Alexa Fluor® imaging assay (TUNEL, Invitrogen, Canada) was employed according to the manufacturer’s instructions. Briefly, wells of a 96-well plate were covered with SWCNT-1 (CB-Ni-Co-Y2O3) suspension in pure ethanol (100 µg/mL) and let dry overnight under the hood. The MC3T3-E1 preosteoblasts were then seeded on SWCNTs at 10 000 cells/cm² and incubated at 37 °C under a humidified 5 % CO₂ atmosphere for 24 h. The cells were fixed using 3 % (w/v) paraformaldehyde in PBS for
15 min at RT, permeabilized using 0.25 % (v/v) Triton X100 in PBS for 20 min at RT and washed twice with milliQ water. A DNase I solution was used as a positive control to provide a positive TUNEL reaction by creating strand breaks in the DNA. The terminal deoxynucleotidyl transferase (TdT) reaction buffer was added to each well and the plate was incubated for 10 min at RT. Thereafter, the cells were incubated with the TdT reaction cocktail for 60 min at 37 °C and washed twice with 3 % (w/v) BSA in PBS for 2 min followed by immediate addition of the Click-iT® reaction cocktail to the wells and incubation for 30 min at RT while protected from light. Each well was rinsed with 3 % (w/v) BSA in PBS for 5 min. Hoechst 33342 solution was added to stain the DNA and cells were incubated for 15 min at RT in the absence of light. Imaging was performed using an Eclipse TE2000-S microscope equipped with a 20x objective and a Retiga 1300R camera (Nikon).

5.4.7 Statistical analysis

All statistical computations were performed with the Statistical Analysis System (SAS-9.2, Cary, NC, USA) using Tukey-Kramer multiple comparison tests (ANOVA). Values were considered significantly different if p<0.05.

5.5 Results

5.5.1 Material characterization

The HRSEM images of the SWCNT samples synthesized using three catalyst mixtures and labeled as SWCNT-1, SWCNT-2 and SWCNT-3 (Figure 5.1) revealed the presence of SWCNT bundles along with carbonaceous impurities in all experimental conditions. The morphology of all SWCNTs seemed similar regardless of the different catalyst mixtures used for their production. Furthermore, the mean length of the individual SWCNTs have been determined in our previous published work to be between 1 to 2 μm [Kim et al., 2009b].
Raman analyses (Figure 5.2) also revealed that all SWCNT samples contained the characteristic features of SWCNTs as shown by radial breathing mode (RBM) region (100-300 \( \text{cm}^{-1} \)), D-band (1350-1450 \( \text{cm}^{-1} \)) and G-band (1500-1600 \( \text{cm}^{-1} \)) [Dresselhaus et al., 2005]. RBM and D-band regions were further studied in Figure 5.2 (b) and (c). Raman peaks in the RBM region of the three SWCNT samples showed the same diameter distribution with the majority of the CNTs having a diameter size of 1.2 nm. This diameter was calculated according to the equation of \( \omega = 12.5 + 223.5 / d \) [Bachilo et al., 2002], where \( d \) is the tube diameter in nanometers, and \( \omega \) is the wave number in \( \text{cm}^{-1} \). Furthermore, the G/D intensity ratio was substantially greater for the SWCNT-1 synthesized using the mixture CB-Ni-Co-Y\(_2\)O\(_3\) than SWCNT-2 and SWCNT-3 synthesized with CB-Ni-Y\(_2\)O\(_3\) and CB-Ni-Mo-Y\(_2\)O\(_3\), respectively. These results indicated the higher quality of SWCNT-1 produced using CB-Ni-Co-Y\(_2\)O\(_3\).
The Vis-NIR region of the optical absorption in solution phase was studied to compare the purity of SWCNTs (Figure 5.3, a-c). The results indicated the interbands light absorption of SWCNT in $S_{11}$, $S_{22}$ and $M_{11}$ regions for all three SWCNT samples. As reported by Itkis et al. (2003), the purity can only be evaluated through $S_{22}$ band using the absorption spectrum of SWCNT since it is much less sensitive to the environment [Itkis et al., 2003]. The highest purity of SWCNT sample is characterized by the highest Itkis value. The ratio of the area under the $S_{22}$ curve (Figure 5.3, a-c) before and after the baseline subtraction (Itkis value) was about 0.021, 0.0147 and 0.012 for SWCNT-1, SWCNT-2 and SWCNT-3, respectively. The results of UV-Vis-NIR spectroscopy were therefore in accordance with those of the Raman spectroscopy, confirming a better quality and purity of SWCNT-1 produced with the CB-Ni-Co-Y$_2$O$_3$ than those produced by CB-Ni-Y$_2$O$_3$ and CB-Ni-Mo-Y$_2$O$_3$.

The weight loss percent (TGA) of the SWCNT samples as a function of temperature is depicted in Figure 5.3 (d). Since the high TG operating temperature of over 800 °C in the oxidizing air environment insures the removal of carbonaceous species including SWCNTs from the sample, the residual mass can be attributed to the catalyst content of each sample [Shahverdi and Soucy, 2012] which was 21.2 wt % for SWCNT-1, 15.6 wt % for SWCNT-2, and 16.2 wt % for SWCNT-3. The higher amount of catalysts found in the SWCNT-1 produced by CB-Ni-Co-Y$_2$O$_3$ mixture indicates the presence of more amorphous carbon before thermal-oxidation process.
Figure 5.3 Absorption spectra in the range of the $S_{22}$ interband transition before (bottom) and after (top) baseline subtraction of (a) SWCNT-1 (b) SWCNT-2 and (c) SWCNT-3. (d) TG graphs of SWCNT-1 (black line), SWCNT-2 (dash line), and SWCNT-3 (dot line).

XRD results (Table 5.2) revealed the presence of Ni, Co and $Y_2O_3$ in the SWCNT-1 produced by CB-Ni-Co-$Y_2O_3$, while only Ni and $Y_2O_3$ were detected in the SWCNT-2 synthesized with CB-Ni-$Y_2O_3$. However, only pure Ni and $Y_2O_3$ were detected in the SWCNT-3 produced by CB-Ni-Mo-$Y_2O_3$ and Mo was only found in the form of MoC compound. $YC_2$ and NiO were also detected in all SWCNT samples as a by-product of SWCNT synthesis through induction thermal plasma.

The XPS analyses (Table 5.2) showed that C and O were the main components of the outermost surface of all the three SWCNT samples.
Table 5.2 Composition and surface chemistry of SWCNTs.

<table>
<thead>
<tr>
<th>SWCNT</th>
<th>Composition</th>
<th>Surface elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWCNT-1</td>
<td>Ni, Co, Y₂O₃, YC, NiO</td>
<td>C, O</td>
</tr>
<tr>
<td>SWCNT-2</td>
<td>Ni, Y₂O₃, YC, NiO</td>
<td>C, O</td>
</tr>
<tr>
<td>SWCNT-3</td>
<td>Ni, Y₂O₃, MoC, YC, NiO</td>
<td>C, O</td>
</tr>
</tbody>
</table>

5.5.2 Effect of SWCNTs on cell morphology

**SWCNTs seeded on the MC3T3-E1 preosteoblasts attached to PS**

Phase contrast optical microscopy images of the MC3T3-E1 preosteoblasts after 24 h exposure to SWCNT-1 suspensions in cell culture medium supplemented with FBS are presented in Figure 5.4 (a-c). Only the results of cells in contact with SWCNT-1 are shown due to the similarity of the morphology of the cells after exposure to the all three SWCNTs. The untreated control cells (CTL) exhibited a well spread morphology almost reaching the confluence after incubation for 24 h (Figure 5.4 a). In cultures exposed to 0.1 µg/mL of SWCNT-1, the MC3T3-E1 preosteoblasts remained also well spread with the morphology and the number similar to those of the untreated cells (Figure 5.4 b). No rounded cells were observed after 24 h exposure to 100 µg/mL of SWCNT-1. However, the number of attached MC3T3-E1 preosteoblasts in these cultures seemed to decrease slightly in comparison to untreated cells (Figure 5.4 c).

**MC3T3-E1 preosteoblasts seeded on SWCNT-covered PS**

As described above, culture plates were covered by SWCNT suspensions in ethanol and the cells were seeded on the SWCNT-covered plates after vaporization of ethanol. The morphology of the cells was examined after 24 h exposure (Figure 5.4 d-f). Ethanol treatment affected neither the ability of the MC3T3-E1 preosteoblasts to attach to the surface nor their morphology (CTL) (Figure 5.4 d). The cells seeded after ethanol vaporization exhibited a well spread morphology and almost reached the confluence after 24 h incubation. The MC3T3-E1 preosteoblasts seeded on dishes covered by 0.1 µg/mL of SWCNT-1 (Figure 5.4 e) were also well spread after 24 h incubation with the number of cells similar to the untreated cells. Moreover, the MC3T3-E1 preosteoblasts seeded on dishes covered by 100 µg/mL of SWCNT-1 (Figure 5.4 f) were able to attach and had morphology similar to the untreated cells. No
rounded cells were observed in the cultures after 24 h incubation indicating that incubation with SWCNT samples neither strongly affected the morphology of MC3T3-E1 preosteoblasts nor induced any cell detachment.

![Figure 5.4 Morphology of MC3T3-E1 preosteoblasts after 24 h incubation with or without SWCNTs visualized by light microscopy (magnification 10x) bar = 100 μm. (a) CTL cells seeded on culture treated PS dish, (b) and (c) cells treated with 0.1 and 100 μg/mL of SWCNT-1 suspended in cell culture medium supplemented with 10 % FBS respectively. (d) CTL cells seeded on culture dish treated with ethanol. (e) and (f) cells seeded on SWCNT-1 covered dishes, 0.1 and 100 μg/mL in ethanol respectively.]

5.5.3 Effect of SWCNTs on the cell viability and membrane integrity

SWCNTs seeded on MC3T3-E1 preosteoblasts attached to PS

The effect of SWCNTs produced by the three catalyst mixtures on the cell viability was initially evaluated by MTS (Figure 5.5 a) and NR (Figure 5.5 b) assays. The amount of LDH release as a result of membrane damage was also analyzed in the supernatants of MC3T3-E1 preosteoblast cultures treated with different SWCNT samples (Figure 5.5 c). MTS assay showed that incubation of cells with suspensions of SWCNTs for 24 h decreased their viability in a dose-dependent manner irrespective of the catalyst mixtures used. For example, mitochondrial enzymatic activity which is proportional to the number of viable cells was
significantly lower (p<0.05) in cultures exposed to the highest (i.e. 100 µg/mL) concentration of all SWCNTs than that in the cultures treated with the lowest concentration (i.e. 0.1 µg/mL). The highest concentration (i.e. 100 µg/mL) of all three SWCNTs caused more than 50% loss of mitochondrial enzymatic activity in MC3T3-E1 preosteoblasts after 24 h incubation. The results obtained with LDH assay were in accordance with those obtained with MTS assay. All three SWCNT samples induced a significant LDH release from the MC3T3-E1 preosteoblasts in a dose-dependent manner after 24 h incubation indicating a considerable cell membrane damage and cellular death particularly in cultures treated with high concentrations (i.e. 50 and 100 µg/mL). In contrast, there was a discrepancy between the results obtained with the NR assay and those observed with MTS and LDH assays. No significant reduction in the NR incorporation was observed after 24 h incubation even at the highest concentration of SWCNT-1 and SWCNT-2. A slight reduction in the NR incorporation was only observed in MC3T3-E1 preosteoblasts treated with 10 and 50 µg/mL of SWCNT-3 synthesized using CB-Ni-Mo-Y₂O₃ mixture. However, surprisingly the highest concentration (i.e. 100 µg/mL) of SWCNT-3 did not induce any significant decrease in the NR uptake. Therefore, NR assay does not seem to be a reliable method for studying the viability of MC3T3-E1 preosteoblasts treated with SWCNTs.
Figure 5.5 Viability of MC3T3-E1 preosteoblasts after 24 h incubation with or without SWCNTs (cells under the SWCNTs) examined by (a) MTS and (b) NR assays. (c) LDH leakage from MC3T3-E1 preosteoblasts after 24 h incubation with SWCNTs (cells under the SWCNTs) examined by LDH assay. Absorbance was recorded at 490 nm for MTS and LDH assays and at 540 nm for neutral red assay. Absorbance was normalized to absorbance of CTL cells. Results are expressed as mean ± SD of at least two independent experiments performed in triplicate. (*) shows statistically significant difference compared to untreated control cells (p<0.05).
MC3T3-E1 preosteoblasts seeded on SWCNT-covered PS

In order to understand whether the reduction of the cell viability observed with different SWCNTs was due to cell membrane damages possibly made by the weight of SWCNTs, the MC3T3-E1 preosteoblasts were seeded on SWCNTs. MTS, and LDH assays were again performed on cells seeded on SWCNTs with different concentrations for 24 h (Figure 5.6 a and b). Due to non-reproducible results of NR assay, no further results of this technique were presented in this study. MTS assay (Figure 5.6 a) showed similar mitochondrial enzymatic activity in the MC3T3-E1 preosteoblasts in contact with SWCNT-2 and SWCNT-3 synthesized using CB-Ni-Y_2O_3 and CB-Ni-Mo-Y_2O_3 respectively suggesting no decrease in cell viability compared to untreated cells. In contrast, SWCNT-1 synthesized using CB-Ni-Co-Y_2O_3 showed a slight decrease in the cell viability at 50 and 100 μg/mL in comparison to the untreated cells (p<0.05) which could be due to the higher catalyst content of SWCNT-1.

LDH assay (Figure 5.6 b) showed no LDH enzyme leakage from cells seeded on any of the SWCNT samples despite the fact that MTS assay showed a slight reduction in cell viability in cultures seeded on SWCNT-1. These results suggest that no significant membrane disruption occurred in the cells that were seeded on the SWCNTs. The reduction of the mitochondrial enzymatic activity without cell membrane damage might be due to apoptosis [Denning et al., 2002; Newmeyer et al., 1994]. Therefore, TUNEL assay was performed to figure out whether this loss of mitochondrial enzymatic activity was resulted from apoptosis (Figure 5.6 c). However, no apoptotic cells were detected in cultures seeded on SWCNT-1 (100 μg/ml in ethanol) for 24 h.
Figure 5.6 (a) Viability of MC3T3-E1 preosteoblasts seeded on SWCNTs for 24 h examined by MTS assay. (b) LDH leakage from MC3T3-E1 preosteoblasts seeded on SWCNTs for 24 h examined by LDH assay. Absorbance was recorded at 490 nm and normalized to absorbance of CTL cells. Results are expressed as mean ± SD of at least two independent experiments performed in triplicate. (*) shows statistically significant difference compared to untreated control cells (p<0.05). (c) TUNEL apoptosis assay performed on cells seeded on SWCNT-1 synthesized using (CB-Ni-Co-Y$_2$O$_3$-100µg/mL in ethanol) for 24 h to distinguish apoptosis. In positive control cells DNase I solution has created DNA strand breaks and nuclei are stained red. In negative control nuclei of cells are stained blue by Hoechst 33342 solution. Cells were observed under fluorescent microscopy (magnification 20 x), bar = 100 µm. Results are representative of two independent experiments.
5.5.4 Effect of SWCNTs on cell proliferation

SWCNTs seeded on MC3T3-E1 preosteoblasts attached to PS

Results of MTS assay performed on the MC3T3-E1 preosteoblasts (treated with SWCNTs at 40% of confluence) showed a dose-dependent decrease of proliferation 24 and 48 h after adding SWCNTs on the cells (Figure 5.7 a). The proliferation was decreased more than 50% in cultures treated with 100 μg/mL of all SWCNTs. Moreover, in cultures treated with each of the three SWCNT samples with concentrations higher than 10 μg/mL, cellular growth was prevented between 24 and 48 h in comparison to the untreated cells. The effects of all SWCNT samples on the proliferation of MC3T3-E1 preosteoblasts were similar despite different catalyst mixtures used for their production.

MC3T3-E1 preosteoblasts seeded on SWCNT-covered PS

The results of MTS assay on 40% confluent cells seeded on the SWCNTs (Figure 5.7 b) showed a very slight dose-dependent decrease of proliferation. At the highest concentration tested, only about 20% reduction (p <0.05) was observed in cellular proliferation with all the SWCNT samples which was much lower than the reduction of proliferation observed in cultures treated with the same SWCNTs on top of the cell. Moreover, comparing the growth of the cells after 24 and 48 h, a significant increase in the growth was observed between 24 and 48 h for all experimental conditions (p <0.05).
5.5.5 Effect of SWCNTs on DNA and actin cytoskeleton of cells

In order to understand whether the reduced proliferation observed in MC3T3-E1 preosteoblasts treated with SWCNTs was related to the reorganization of F-actin cytoskeleton inside the cells as reported by Holt et al. [Holt et al., 2010], the F-actin was labeled with rhodamine-phalloidin in the cells seeded either on or under the SWCNTs. Moreover, changes in the nucleus such as chromatin condensation and DNA fragmentation were analyzed using DAPI staining. Regardless of the catalyst mixture used for production of the SWCNT samples and the way cells were treated with SWCNTs (i.e. putting SWCNT suspensions on the cells or seeding cells on SWCNTs), nuclei and F-actin cytoskeleton of the remaining attached cells
were not strongly affected by SWCNTs after 24 h incubation (Figure 5.8). The untreated cells along with those treated with the highest concentration (i.e. 100 µg/mL in culture medium supplemented with 10 % FBS) of all three SWCNT samples suspended in cell culture medium and also cells seeded on different SWCNT samples (100 µg/mL in ethanol) were well spread, had organized F-actin cytoskeleton, and showed no signs of chromatin condensation.

Figure 5.8 DNA and F-actin cytoskeleton of MC3T3-E1 preosteoblasts after 24 h incubation with SWCNTs. DNA and filamentous actin cytoskeleton of MC3T3-E1 preosteoblasts seeded either under or on the SWCNTs (100 µg/mL) were studied by DAPI and rhodamine-phalloidin staining respectively. Cells were visualized under fluorescent microscopy (magnification 60 x) bar = 100 µm. Results are representative of two independent experiments.

5.6 Discussion

It has been reported that CNTs interact with some of the colorimetric and fluorescent dyes used in the toxicity assessments [Casey et al., 2007; Worle-Knirsch et al., 2006]. Therefore, it is mandatory to apply various cytotoxicity assays which assess the impact of these materials on different cell organelles in parallel to be able to conclude correctly about the cytotoxicity of the tested SWCNTs. In the present study, therefore, three cytotoxicity assays were performed
to evaluate the effect of RF induction thermal plasma grown SWCNTs on the cellular viability. MTS assay was employed for analysis of the cell viability by measuring reduction in the metabolic activity of cells which is an early indication of cellular damage [Baltrop et al., 1991; Cory et al., 1991]. Moreover, MTS assay is proven to be one of the most accurate assays in determining the cytotoxicity of carbonaceous nanomaterials [Monteiro-Riviere et al., 2009]. NR assay which has a very sensitive and easily quantifiable procedure is based on the ability of viable cells to incorporate the NR dye in their lysosomes [Borenfreund and Puerner, 1984; Repetto et al., 2008]. LDH assay is applied to estimate the amount of seriously injured or dead cells. In this assay, LDH enzyme leakage which is resulted from the loss of membrane integrity is measured [Mitchell et al., 1981].

In agreement with previous reports [Davoren et al., 2007], the results of the cytotoxicity tests in this study revealed an obvious interference of the SWCNTs with NR assay. Davoren et al. had previously reported a significant interference with NR dye when assessing the viability of A549 cells, a human lung carcinoma epithelial cell line, after exposure to SWCNT suspensions in culture medium supplemented with 5 % FBS [Davoren et al., 2007]. In the present study, fluctuations were observed in the results obtained with NR assay when cells were treated with SWCNTs, particularly when suspended in αMEM supplemented with 10 % FBS. It seems that NR dye could be adsorbed by SWCNTs resulting in unexpected absorption data despite the fact that a “blank” condition containing SWCNTs without cells was considered in this study to eliminate this type of background. Therefore, the results of NR assay cannot be conclusive alone and must be confirmed by other cytotoxicity assays due to the adsorption of the NR dye to the SWCNT material.

In contrast to NR assay, MTS and LDH assays revealed constant and dependable results confirming each other and indicating no interference between the SWCNTs and these two techniques.

Many previous studies have attributed the cytotoxicity of CNTs to the presence of metallic catalysts [Cui et al., 2005; Fiorito et al., 2006a; Jia et al., 2005; Kagan et al., 2006; Shvedova et al., 2003]. Metallic impurities are shown to be responsible for the redox properties of CNTs [Banks et al., 2006; Kruusma et al., 2007; Pumera et al., 2009; Pumera and Iwai, 2009; 128
Moreover, there is a huge amount of literature on the toxicity of Ni, Co, Mo, and Y$_2$O$_3$ [Ada et al., 2010; Andelman et al., 2010; Benson et al., 1986; Cangul et al., 2002; Colognato et al., 2008; Freitas et al., 2010; Kolesarova et al., 2010; Rudolf and Cervinka, 2010; Sunderman, 1976]. Besides, our previous study has shown the cytotoxicity of metallic nanoparticles synthesized by RF induction thermal plasma process under the same operating conditions as SWCNT synthesis [Alinejad et al., 2012]. However, quite similar cytotoxic behavior was observed for all the SWCNTs produced using three different catalyst mixtures. The ability of the MC3T3-E1 preosteoblasts seeded on the SWCNTs to attach properly to the substrate without presence of rounded cells in the cultures was promising since adhesion of osteoblasts to the substrate is considered as a crucial first step for cellular functions such as viability, proliferation, protein synthesis, and mineralization [Kalbacova et al., 2006]. Furthermore, using two strategies for exposing the cells to SWCNTs (i.e. seeding cells either under or on the SWCNTs) made it possible to assess the viability and proliferation of the cells in contact with SWCNTs without mechanical disturbance. Indeed, regardless of the catalyst mixture used for SWCNT production, a reduction of the cell viability was observed when SWCNTs were added on the cells. The leakage of LDH enzyme in these cultures implied that reduction in the viability could be due to the cell membrane disruption. However, when cells were seeded on the SWCNTs their membranes were not damaged and no loss of viability was observed after 24 h incubation. The only exception observed was when the MTS assay showed statistically significant decrease of mitochondrial enzymatic activity in the cells seeded on the higher concentrations of SWCNT-1 synthesized using CB-Ni-Co-Y$_2$O$_3$ without strong damaged cell membrane or apoptosis. This result may be attributed to the higher amount of catalysts present in SWCNT-1 sample as revealed by TGA analysis. It seems that using Ni-Co-Y$_2$O$_3$ as the catalyst mixture resulted in the production of as-produced SWCNT sample with higher amount of amorphous carbon. As mentioned before, in order to increase the purity, the amorphous carbon contents of the SWCNT samples were removed by thermal oxidation after the synthesis process. Therefore, the presence of higher amount of catalyst in SWCNT-1 sample implies higher amount of amorphous carbon before performing thermal oxidation process.
In addition to the results of viability assays, comparing the proliferation of the cells exposed to SWCNTs with two mentioned strategies showed that adding SWCNTs on the cells prevented their proliferation. By contrast, cells seeded on top of the SWCNTs could grow significantly between 24 and 48 h although the mitochondrial enzymatic activity (proportional to the number of viable cells) in the presence of SWCNTs was less than that in the control after 24 h. Therefore, it can be concluded that cytotoxic effects of the RF induction thermal plasma grown SWCNTs suspended in culture medium might be because of a mechanical disturbance of the cell membrane or other possible interactions between the preosteoblasts and these materials. In fact, lipid membrane disturbance has been reported after treating HeLa cells with dispersed SWCNTs [Yaron et al., 2011]. Yaron et al. found that dispersed SWCNTs can penetrate through the first lipid layer inducing some membrane tension [Yaron et al., 2011]. Moreover, dispersed CNTs in evaporating solvents such as ethanol form packed and dense aggregates after evaporation [Li et al., 2006]. Therefore, some changes in the aggregation state of SWCNTs immobilized on polystyrene after ethanol evaporation may explain their poor cytotoxicity in comparison to SWCNTs suspended in culture medium.

The lack of strong toxicity of SWCNTs produced by RF induction thermal plasma might also be explained by the fact that catalytic gas-phase synthesis of CNTs is based on the evaporation of a feedstock material containing a carbon source and catalysts. It has been suggested that most of the catalyst particles become molten at the temperature of the SWCNT synthesis [Harutyunyan et al., 2005]. Dissolution of the carbon precursors in liquid metallic catalysts is followed by precipitation and then formation and growth of SWCNTs [Gorbunov et al., 2002; Moisala et al., 2003]. Once the catalyst particle solidifies, a massive dissociation of the extra carbon dissolved in the particle results in rapid encapsulation of the catalyst particle in a graphitic shell layer [Jouret et al., 2012]. Carbon with Sp² hybridization is a chemically inert material and the only damage it causes to the cells results from the mechanical disturbance [Pumera and Miyahara, 2009]. In the SWCNT synthesis by RF induction thermal plasma process most of the catalyst particles participate in the synthesis either as a nucleation site for SWCNTs growth or in the form of particles encapsulated in a graphitic layer. In fact, HRSEM images of the purified SWCNTs have confirmed the presence of these graphitic shells in our samples (data not shown). A small part of the catalysts will not be treated by plasma and
remain in the final product [Kim et al., 2009a]. Overall, despite the fact that the SWCNT samples tested in the present study contained about 15-20 wt % catalysts, these metallic particles were mostly encapsulated in a graphite shell that limits their cytotoxicity. However, further experiments are required to determine whether these SWCNTs can affect the ability of preosteoblasts to differentiate and function at longer term.

The morphology of CNTs was shown to affect their cytotoxicity as well [Johnston et al., 2010]. Generally, fibers length plays an important role in their potential toxicity [Donaldson et al., 2006]. Long fibers are considered more harmful because not only their clearance by phagocytic cells is more difficult but also they are more stimulatory and biologically active than short fibers in contact with the cells [Brown et al., 2007; Donaldson et al., 2006; Kostarelos, 2008]. Furthermore, CNTs can have aligned or twisted and entangled morphology. Some techniques such as CVD produce aligned CNTs [Hahm et al., 2011] while other techniques such as laser ablation make twisted and entangled CNTs [Kingston et al., 2004]. It is likely that the aligned needle-like CNTs may more easily penetrate into the cell membrane, resulting in greater cell membrane damage and thus cytotoxicity [Zhang et al., 2010]. Therefore, the twisted and entangled morphology of the RF induction thermal plasma grown SWCNT which is not dependent on the metallic catalyst mixture content used for their synthesis seems to be a positive characteristic for them.

5.7 Conclusion

Results show that RF induction thermal plasma has the possibility to apply various metallic particles as catalysts for SWCNT production. Using Co particles in the catalyst mixture results in higher quality SWCNTs. However, the SWCNTs synthesized with this mixture seem to have slightly increased cytotoxicity.

The procedure employed for treating cells with SWCNTs can affect the results and even can mislead experimenters with false conclusions.

Neglecting the loss of viability that might be caused by weight and pressure of the materials on the cells, RF induction thermal plasma grown SWCNTs neither strongly affected the viability nor the proliferation of MC3T3-E1 preosteoblasts. Although still more research is
needed to get further insights into the toxicological profile of these SWCNTs and their impact on the differentiation and function of bone cells, it can be suggested that RF induction thermal plasma grown SWCNTs could be considered as promising CNTs to be studied more deeply for future applications in bone tissue engineering.

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CHAPTER 6 Biocompatibility testing of single-walled carbon nanotubes on murine preosteoblasts: higher osteoblastic differentiation by BMP-9 in comparison with BMP-2

Avant-propos

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Contribution to the document:

This article contributes to the thesis by demonstrating the effect of the purified SWCNTs on the proliferation, differentiation, and function of bone forming cells on the basis of a hypothesis that purified RF induction thermal plasma grown SWCNTs have an influence on
the ability of the bone cells to respond to growth factors and the induced differentiation state of these cells. As a prerequisite we characterized the purified SWCNT sample in terms of morphology and composition and showed that up to 100 μg/mL of these purified SWCNTs had no effect on the cell viability and attachment after 72 h incubation. Investigating the effect of SWCNTs and BMPs combination on the differentiation and function of bone forming cells provided the opportunity to study the bone cell responses to these purified SWCNTs in the conditions that are probable when applying these materials in bone applications.

Yasaman Alinejad performed the SWCNT synthesis and purification experiments under the supervision of Prof. Gervais Soucy and Prof. Nathalie Faucheux. Yasaman Alinejad also performed the biology related experiments with Alex Daviau and Olivier Drevelle under the supervision of Prof. Nathalie Faucheux. The statistical analyses as well as drafting the manuscript were performed by Yasaman Alinejad under the supervision of Prof. Nathalie Faucheux and Prof. Gervais Soucy.

6.1 Résumé

Les nanotubes de carbone (CNTs) ont été utilisés dans les applications orthopédiques en raison de leurs propriétés mécaniques exceptionnelles. Cependant, l'influence des nanotubes de carbone sur le comportement des cellules osseuses et leur capacité à répondre aux facteurs de croissance tels que les protéines morphogénétiques osseuses (BMP) reste mal connue. Par conséquent, dans la présente étude, les CNTs mono-parois (SWCNTs) ont été synthétisés par un procédé de plasma thermique inductif et purifiés en utilisant une procédure en plusieurs étapes. Ensuite, leur influence sur la viabilité et la prolifération des préostéoblastes MC3T3-E1 a été déterminée en mesurant l'activité enzymatique mitochondriale et en réalisant des essais TUNEL. L'impact des SWCNTs sur l'activation des Smads et la différenciation cellulaire induites par 1,92 nM de BMP-2 et BMP-9 a également été étudié en utilisant des analyses western blot et RT-PCR. Le prétraitement des cellules par les SWCNTs a accéléré l'activation des Smad1/5/8 induite par les BMP-2 et BMP-9 (15 min), n'a pas réduit la viabilité des préostéoblastes mais a légèrement affecté leur prolifération à 48 h. De plus, après 72 h d'incubation avec la BMP-2 ou la BMP-9, les préostéoblastes MC3T3-E1 prétraités avec des SWCNTs pendant 24 h pouvaient exprimer des gènes codant pour des marqueurs
ostenogéniques et présentaient une forte activité de la phosphatase alcaline. Fait intéressant, la BMP 9 a favorisé la différenciation des préostéoblastes MC3T3-E1 prétraités par les SWCNTs de manière plus importante que la BMP-2. Par conséquent, la combinaison de la BMP-9 avec les SWCNTs semble être une voie prometteuse pour la régénération osseuse.

6.2 Abstract

Carbon nanotubes (CNTs) have been used in orthopaedic applications because of their exceptional mechanical properties. However, the influence of CNTs on the behaviour of bone-forming cells and on the ability of these cells to respond to growth factors, such as bone morphogenetic proteins (BMPs), remains poorly known. Therefore, in the present study, single-walled CNTs (SWCNTs) were synthesised using an induction thermal plasma process and purified using a multistep procedure. The impact of these purified SWCNTs on the Smad activation, cell proliferation and differentiation, with or without BMP-2 and BMP-9 (1.92 nM), was also studied using western blot, mitochondrial enzymatic activity, TUNEL, RT-PCR and alkaline phosphatase activity analyses. Pre-treatment of MC3T3-E1 preosteoblasts with SWCNTs accelerated the Smad1/5/8 activation, induced by both BMP-2 and BMP-9, within 15 min. It also slightly affected their proliferation at 48 h without apoptosis. Interestingly, at 72 h, BMP-9 favoured the differentiation of MC3T3-E1 preosteoblasts pre-treated with SWCNTs to a larger extent than BMP-2 did. Therefore, the combination of BMP-9 with SWCNTs appears to be a promising avenue for bone applications.

6.3 Introduction

A bone is a dynamic tissue that is continuously remodelled by the activity of osteoclasts and osteoblasts and has substantial capacity for repair and regeneration [Marquis et al., 2009]. Several growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and bone morphogenetic proteins (BMPs, members of the transforming growth factor beta (TGF-β) superfamily), stimulate bone formation and repair [Fernandez-Tresguerres-Hernandez-Gil et al., 2006b]. Thus far, more than 20 BMPs have been identified [Senta et al., 2009]. Most of these BMPs (BMP-2, BMP-6, BMP-7 and BMP-9) are involved in the differentiation of mesenchymal stem cells (MSCs) into the osteoblast lineage and play a
crucial role in the final differentiation of preosteoblasts into mature osteoblasts [Luu et al., 2007; Senta et al., 2009].

BMPs act on bone cells by binding to two type I and two type II transmembrane serine/threonine kinase receptors, resulting in the phosphorylation of type I receptors by the type II receptors and consequently the activation of the canonical Smad pathway [Senta et al., 2009]. The combination of two phosphorylated receptor-regulated Smads (R-Smads) made of Smad1, Smad5, and Smad8 forms a complex with Smad4, a common-partner Smad (Co-Smad). The translocation of this complex into the nucleus regulates the transcription of target genes encoding markers of osteogenic differentiation, such as osterix (Osx), alkaline phosphatase (ALP), and osteocalcin (OC) [Senta et al., 2009].

Currently, BMP-2 is the most frequently used BMP in bone healing studies and is approved by the Food and Drug Administration (FDA) for clinical use in the USA [Bessa et al., 2008; Senta et al., 2009]. However, BMP-9 appears to be a better osteogenic factor than BMP-2 because it induced a greater differentiation of MSCs into osteoblasts in vitro [Luu et al., 2007] and a more effective bone regeneration in vivo [Kang et al., 2004]. Moreover, carbon nanotubes (CNTs), which are one-dimensional macromolecules of carbon, can be used in the design of biomimetic materials for applications in bone repair and tissue engineering because of their tremendous strength, ultralight weight, high stability and flexibility [Aoki et al., 2007; Jamilpour et al., 2011; Usui et al., 2008; Venkatesan and Kim, 2012]. However, controversy still exists regarding the biocompatibility of CNTs in bone applications. Some in vitro studies reported that CNTs are cytotoxic to bone cells [Liu et al., 2010; Narita et al., 2009; Tutak et al., 2009], while others suggested that CNTs are excellent substrates for bone cell growth and differentiation [Akasaka et al., 2010; Kalbacova et al., 2007; Shi et al., 2007]. These contradictory findings could result from the distinct properties of the CNTs used in these studies, such as different impurity contents, which can have a substantial impact on their cytotoxicity [Alinejad et al., 2012].

In addition, the use of CNTs in bone requires a better understanding of their effect on the ability of bone cells to respond to their environment, especially growth factors. However, only a few studies have investigated the effect of CNTs in combination with BMP-2 on bone cells
in recent years [Li et al., 2012; Nayak et al., 2010; Van Der Zande et al., 2010]. Furthermore, up to now, there is still no report, to our knowledge, on the effect of CNTs on bone cell responses to BMP-9.

In the present study, we have therefore synthesised SWCNTs using radio frequency (RF) induction thermal plasma, purified them as previously described [Kim et al., 2009a], and characterised their surface chemistry by X-ray photoelectron spectroscopy (XPS). The effects of SWCNTs on the behaviour of MC3T3-E1 preosteoblasts, used as model cells since their differentiation is similar to human cells [Bergeron et al., 2009], with or without an equimolar concentration (1.92 nM) of BMP-2 or BMP-9 were also determined. This concentration of BMPs was selected since it has been shown to be effective for inducing osteoblastic differentiation in MC3T3-E1 preosteoblasts [Bergeron et al., 2009]. First, the phosphorylation state of Smad1/5/8 proteins in MC3T3-E1 preosteoblasts pre-treated with or without SWCNTs and stimulated with BMP-2 or BMP-9 were determined by western blot analyses. We also used immunolabelling to follow the translocation of the phosphorylated Smad1/5/8 to the nuclei of these cells. The proliferation of MC3T3-E1 preosteoblasts pre-treated with or without SWCNTs and stimulated with BMP-2 or BMP-9 was evaluated within 48 h, and the apoptosis was checked using a TUNEL assay. Finally, the differentiation of MC3T3-E1 preosteoblasts was verified by quantifying the expression of genes encoding osteogenic markers and measuring the ALP activity.

6.4 Materials and methods

6.4.1 SWCNT synthesis and purification

SWCNT materials were synthesised using an RF induction thermal plasma process as described previously [Kim et al., 2009b]. Briefly, the feedstock material, containing carbon black (CB) as the carbon source and a ternary mixture of nickel (Ni), cobalt (Co), and yttrium oxide (Y$_2$O$_3$) as catalysts (CB-Ni-Co-Y$_2$O$_3$: 87.2-2.6-2.6-7.5 wt %), was continuously introduced through a water-cooled probe placed at the centre of a TEKNA PS-50 plasma torch (Tekna Plasma Systems, Inc. Canada) into the synthesis system consisting of a tubular reactor and a filtration system with three metallic filters. A mixture of argon and helium was used to
generate the plasma, and the synthesis process was performed with 40 kW plate power and 67 kPa operating pressure. The sheet-like as-produced SWCNT material was powdered using 900, 250, and 90 μm sieves. A multistep purification procedure previously developed by our group [Kim et al., 2009a] was used to purify the SWCNT sample through amorphous carbon removal by partial thermal oxidation at 350 °C and metallic catalyst content minimization by treatment with boiling nitric acid (7 M HNO₃) and centrifugation.

6.4.2 Surface chemistry analysis of SWCNTs

XPS (Axis UltraDLD spectrometer, monochromatic Al-Kα (hv=1 486.69 eV) source at 225 W and photoelectron collection angle of 52.5 °) was performed to detect the elements present on the surface of the SWCNTs. The energy resolution of the system (i.e., source and analyser) was set to 0.3 eV. The elemental composition of the analysed surface areas was obtained from survey spectra collected at a pass energy of 160 eV.

6.4.3 Cell experiments

Cell culture

Murine calvarial MC3T3-E1 preosteoblasts, subclone 14, (CRL-2594™, ATCC®, USA) were grown in minimum essential medium (MEM) alpha (αMEM, Gibco®, USA) without ascorbic acid, supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS, Wisent, Canada), 100 U/mL penicillin (Invitrogen, Canada) and 100 μg/mL streptomycin (Invitrogen, Canada) at 37 °C under a humidified 5 % CO₂ atmosphere. Cell passage was performed using 0.25 % trypsin-EDTA 1X (Invitrogen, Canada). The cells used for experiments were between passages 3 and 13.

Proliferation of SWCNT-treated MC3T3-E1 preosteoblasts after stimulation with BMPs

The SWCNTs were sterilised in an oven at 180 °C for 3 h. SWCNT suspensions were made in αMEM supplemented with 10 % (v/v) FBS. Before use, the suspensions were placed in an ultrasonic bath for 20 min and were shaken using a vortex mixer. MC3T3-E1 preosteoblasts were seeded in 24-well plates (5 000 cells/cm²) and allowed to attach until reaching ~ 40 % confluence. The cells were then incubated with or without SWCNTs (100 μg/mL; 500 μL per
well) for 4 h. A blank containing the same amount of SWCNTs (100 μg/mL) but no cells was also considered. Next, the cells were stimulated with an equimolar concentration (1.92 nM) of BMP-2 and BMP-9 (R&D Systems, USA) diluted in αMEM and incubated for 24 and 48 h. Cultures treated with the same volume of the medium (αMEM supplemented with 10 % (v/v) FBS) with or without BMP-2 and BMP-9 were used as controls. The proliferation of cells was measured using an MTS assay according to the manufacturer’s instructions (Cell Titer 96® AQueous Non-Radioactive Cell Proliferation, Promega, USA). Briefly, after 24 or 48 h of incubation with SWCNTs, the MC3T3-E1 preosteoblasts were rinsed with sterile phosphate buffered saline (PBS, pH 7.4). Then, 300 μL of culture medium (αMEM supplemented with 10 % (v/v) FBS) and 60 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent were added to each well in the absence of light, and the plate was incubated for 2 h at 37 °C. Subsequently, the supernatants were transferred to a 96-well plate, and the optical density was measured at 490 nm using a microtiter plate reader (Bio-Tek, USA).

**TUNEL apoptosis assay**

To label apoptotic cells, a Click-iT® TUNEL Alexa Fluor® imaging assay (TUNEL, Invitrogen, Canada) was employed according to the manufacturer’s instructions. Briefly, MC3T3-E1 preosteoblasts were seeded in 96-well plates (5 000 cell/cm²). After 24 h of incubation, the cells were incubated with or without SWCNTs (100 μg/mL) for 4 h. Next, the MC3T3-E1 preosteoblasts were stimulated with BMP-2 or BMP-9 (1.92 nM) and incubated for 48 h. The cells were then fixed with 3 % (w/v) paraformaldehyde in PBS for 15 min at RT, washed and permeabilised using 0.25 % (v/v) Triton X100 in PBS for 20 min at RT. A DNase I solution, which creates strand breaks in DNA, was used as a positive TUNEL control. The cells were incubated with the terminal deoxynucleotidyl transferase (TdT) reaction buffer for 10 min at RT and with the TdT reaction cocktail for 60 min at 37 °C. The cells were washed twice with 3 % (w/v) bovine serum albumin (BSA) in PBS for 2 min followed by the immediate addition of the Click-iT® reaction cocktail to the wells and incubation for 30 min at RT while protected from light. Each well was rinsed with 3 % (w/v) BSA in PBS for 5 min. Hoechst 33342 solution was added to stain the DNA, and the cells were incubated for 15 min.
at RT in the absence of light. Imaging was performed using an Eclipse TE2000-S microscope equipped with a 40X objective and a Retiga 1300R camera (Nikon).

**Western blot analyses of the Smad pathway**

For these experiments, MC3T3-E1 preosteoblasts were seeded in 6-well plates (15 000 cell/cm²) and incubated at 37 °C under a humidified 5 % CO₂ atmosphere until reaching ~ 80 % confluence. Afterwards, the cells were incubated with or without an SWCNT suspension (100 μg/mL) at 37 °C for 24 h and then stimulated for 15 min to 4 h with an equimolar concentration of BMP-2 or BMP-9 (1.92 nM). The cells were washed three times with 1 mM orthovanadate (Sigma-Aldrich, Canada) in PBS and lysed at 4 °C in 150 μL of 50 mM Tris-HCl at pH 7.4 containing 0.1 % (v/v) SDS, a complete mini-protease inhibitor cocktail (Roche Diagnostics, USA) and 1 mM orthovanadate. Using SDS-PAGE, equal amounts of the cell lysate proteins were separated and then transferred to nitrocellulose membranes using the Transblot® Semi-Dry electrophoretic transfer cell (Bio-Rad Laboratories, Canada). The transfer efficiency was confirmed by staining the membranes with Ponceau red (Sigma-Aldrich, Canada). The membranes were washed three times with 0.1 % (v/v) Tween 20 in PBS, and protein bands were revealed by incubation with primary rabbit antibodies raised against phosphorylated Smad1 (Ser⁴⁶³/⁴⁶⁵)/Smad5 (Ser⁴⁶³/⁴⁶⁵)/Smad8 (Ser⁴²⁶/⁴²⁸) (diluted 1:1 000) and total Smad1/5/8 (diluted 1:1 000) (both phosphorylated and total Smad1/5/8 were purchased from Cell Signalling Technology, Canada). The membranes were then washed three times with Tween 20 (0.1 % (v/v)) in PBS, and antigen–antibody complexes were detected by incubating them with peroxidase-conjugated anti-rabbit antibodies diluted 1:20 000 (Sigma, USA). All antibodies were diluted in PBS containing 0.1 % (v/v) Tween 20 and 5 % (w/v) skimmed milk (Sigma-Aldrich, Canada). Immunoreactive bands were visualised using chemiluminescence (ECL Plus™, GE Healthcare, UK) and exposure to X-ray film (Thermo Scientific, USA).

**Immunolabelling of phosphorylated Smad1/5/8**

Cells were seeded in 35 mm×10 mm cultures dishes (15 000 cell/cm²) and incubated until reaching ~ 80 % confluence. The cells were then incubated with or without an SWCNT suspension (100 μg/mL) for 24 h followed by stimulation with BMP-2 or BMP-9 (1.92 nM)
for 1 h. Next, the cells were fixed, permeabilised and incubated for 30 min at 37 °C with 1 % BSA (w/v) in PBS to block non-specific binding sites. The cells were immunostained by incubation with primary rabbit antibodies against phosphorylated Smad1 (Ser\(^{463/465}\))/Smad5 (Ser\(^{463/465}\))/Smad8 (Ser\(^{426/428}\)) (diluted 1:50). Bound primary antibodies were visualised by incubation with FITC-conjugated anti-rabbit IgG secondary antibodies (diluted 1:150, Sigma-Aldrich). DNA was stained with DAPI (1 µg/mL) diluted in PBS. The cells were then washed and visualised with an Eclipse TE2000-S microscope with a 20X objective and a Retiga 1300R camera.

**Analysis of mRNA using real-time polymerase chain reaction**

MC3T3-E1 preosteoblasts were seeded in 6-well-plates (10,000 cell/cm\(^2\)) and allowed to attach and grow until reaching ~ 80 % confluence. Afterwards, the cells were incubated with or without an SWCNT suspension (100 µg/mL) for 24 h. Following this incubation time, the cells were stimulated with an equimolar concentration of BMP-2 or BMP-9 (1.92 nM) for 24 and 72 h. The total RNA was extracted from the cells using an RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Canada). The extracted RNA samples were quantified with a GeneQuant pro (Biochrom, UK) UV spectrophotometer at 260/280 nm. An aliquot of RNA (1 µg) was treated with DNase I, and the first-strand cDNA was synthesised using oligo(dT) primers and Superscript II reverse transcriptase. Quantitative polymerase chain reaction (qPCR) was performed, in triplicate, on an iQ™ Real-Time PCR detection system (Bio-Rad Laboratories, Canada) in a 25 µL volume using iQ™ SYBR® Green SuperMix (Bio-Rad Laboratories, Canada). Detailed information about the thermocycling conditions was described previously [Bergeron *et al.*, 2009].

Specific primers for murine Gapdh (Mm_GAPDH_3_SG), Dlx5 (Mm_DLX5_1_SG), Runx2 (Mm_RUNX2_1_SG), Osx (Mm_SP7_1_SG), OC (Mm_BGLAP_1_SG) and ALP (Mm_ALPL_2_SG) were purchased from Qiagen (Canada). Reactions that did not reach the threshold signal value within the first 30 cycles (Cycle threshold, Ct ≥ 30) were discarded. All assays were normalised to Gapdh and analysed using the 2\(^{-ΔΔCt}\) model. A 4 % (w/v) agarose gel containing ethidium bromide was used to evaluate the quality of the PCR products. The
AmpliSize™ Molecular Ruler 50-2000 bp Ladder (Bio-Rad Laboratories, Canada) was used as a control on each gel. All primers generated the expected single band.

**Evaluation of alkaline phosphatase activity**

For this experiment, MC3T3-E1 preosteoblasts were seeded on 6-well plates (10,000 cells/cm²) and incubated until reaching ~80% confluence. The cells were then incubated with or without an SWCNT suspension (100 μg/mL) for 24 h and then stimulated with an equimolar concentration of BMP-2 or BMP-9 (1.92 nM) for 72 h. The ALP activity was measured with the SensoLyte® pNPP alkaline phosphatase assay kit (Anaspec, USA) according to the manufacturer’s instructions. Briefly, the cells were washed twice with the assay buffer and lysed with 300 μL of lysis buffer (0.2% Triton X100 in assay buffer (v/v)); the lysates were centrifuged at 2500 g. The resulting supernatants were collected, and the ALP activity was measured using the p-nitrophenyl phosphate (pNPP) ALP substrate for 1 h. The optical density was measured at 405 nm using a UV/Vis spectrophotometer. The ALP activity was determined with reference to the ALP activity of a standard curve and then to the total protein amount (Bradford assay).

**Statistical analysis**

All statistical computations were performed with the Statistical Analysis System (SAS-9.2, Cary, NC, USA) using the Tukey-Kramer multiple comparison test (ANOVA). Values were considered significantly different for p<0.05.

6.5 Results

6.5.1 Surface chemistry of the purified SWCNTs

Detailed surface chemistry characteristics of the purified SWCNTs were obtained using XPS (Table 6.1). From a general XPS survey, the main elements on the SWCNT surface were found to be C and O with a total concentration of >99.5 at %. Traces (i.e., <0.5 at %) of N, Y, Ni and Co were also detected. Further information about the surface chemistry of the SWCNTs was obtained from the high resolution spectrum of the C1s line fitted to mixed asymmetric Gaussian-Lorentzian (A-GL) line shape peaks. Five individual shifts were
observed and then interpreted to be sp² carbon (C=C), sp³ carbon (C-C), single C-O bonds and double C=O and COO bonds according to our previous study [Shahverdi et al., 2012]. The total percentage of grafted carbon to oxygen atoms on the surface of SWCNTs was calculated to be 13 at %, and the main functional groups were COO and C-O with 6.8 and 5 at %, respectively.

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6.5.2 Smad1/5/8 pathway activation in MC3T3-E1 preosteoblasts treated with SWCNTs and stimulated with BMP-2 or BMP-9

A concentration of the SWCNTs at 100 μg/mL was selected since it neither affected the viability of the MC3T3-E1 preosteoblasts after 24 h nor their attachment and actin cytoskeletal organization within 72 h (data not shown). To verify the ability of MC3T3-E1 preosteoblasts to respond to BMP-2 or BMP-9 (1.92 nM), western blot analyses of the phosphorylated Smad1/5/8 as well as total Smad (used as control) were performed (Fig. 6.1A).

The phosphorylated Smad1/5/8 and total Smad produced a major band with a molecular weight of ~ 60 kDa (Fig. 6.1A). The similarity of the intensities of the total Smad1/5/8 bands in all experimental conditions indicated equal protein loading. In the absence of BMP-2 and BMP-9, no phosphorylated Smad1/5/8 was detected in the CTL and CNT at any time point. However, in the presence of BMP-2 or BMP-9, a phosphorylated Smad1/5/8 band was detected within 30 min in the MC3T3-E1 preosteoblasts without SWCNT pre-treatment. The
intensities of the phosphorylated Smad1/5/8 bands induced by BMP-2 within 1 h were higher than those induced by BMP-9. Interestingly, in the cells pre-treated with SWCNTs for 24 h, the phosphorylation of Smad1/5/8 occurred 15 min after adding BMP-2 or BMP-9. The phosphorylated Smad1/5/8 bands were also more intense in the cells pre-treated with SWCNTs and then stimulated with BMP-2 in comparison to BMP-9 at the 15 and 30 min time points. In the presence of BMP-2, the intensities of the phosphorylated Smad1/5/8 bands in the MC3T3-E1 preosteoblasts with or without SWCNT pre-treatment were increasing within 1 h and became quite stable between 1 and 4 h. In the cells incubated with BMP-9, with or without SWCNT pre-treatment, the intensities of the phosphorylated Smad1/5/8 bands were low within 1 h. Between 2 and 4 h, however, the intensity increased, and the bands were stable.

To properly deliver its signal, phosphorylated Smad1/5/8 should be translocated to the nucleus [Massague et al., 2005]. Because the phosphorylated Smad1/5/8 in the presence of BMP-2 and BMP-9 was detected in MC3T3-E1 preosteoblasts with or without SWCNT pre-treatment within 30 min, their translocation to the nucleus was probed after 1 h using immunofluorescent labelling (Fig. 2). DAPI staining was also used to confirm the nuclear localisation of the phosphorylated Smad1/5/8. Very slight immunostaining of the phosphorylated Smad1/5/8 was detected in the nuclei of the control cells (CTL and CNT) without BMP-2 or BMP-9. More intense staining of phosphorylated Smad1/5/8 was observed in the nuclei of MC3T3-E1 preosteoblasts after stimulation with BMP-2 or BMP-9 with or without SWCNT pre-treatment. The most intense staining of phosphorylated Smad1/5/8 in the nuclei was observed in the MC3T3-E1 preosteoblasts pre-treated with SWCNTs and incubated with BMP-2. This result is in accordance with western blot analyses that showed an earlier and intense Smad1/5/8 phosphorylation in cells treated by both SWCNTs and BMP-2 (Fig. 6.1B).
Figure 6.1 Phosphorylated Smad1/5/8 in MC3T3-E1 preosteoblasts incubated with or without SWCNTs and stimulated with BMP-2 or BMP-9. (A) Western blot analyses of Smad1/5/8 phosphorylation and total Smad1/5/8 in lysates of MC3T3-E1 preosteoblasts incubated with or without SWCNTs for 24 h and stimulated with BMP-2 or BMP-9 for 15 min to 4 h. The results are representative of three independent experiments. (B) Immunostaining of phosphorylated Smad1/5/8 and DNA labelling in MC3T3-E1 preosteoblasts incubated with or without SWCNTs for 24 h and then stimulated with BMP-2 or BMP-9 for 1 h. Bar =100 μm. The results are representative of two independent experiments.

6.5.3 Proliferation of SWCNT treated MC3T3-E1 preosteoblasts after stimulation with BMP-2 or BMP-9

Because BMP-2 and BMP-9 were able to induce the activation of the canonical Smad pathway in all experimental conditions, we evaluated the proliferation of MC3T3-E1 preosteoblasts incubated with BMP-2 or BMP-9, with or without SWCNT pre-treatment, after 24 and 48 h (Fig. 6.2A). At the 24 h time point, the MTS assay revealed a small but significant reduction
of cell proliferation in all the cultures stimulated with BMP-2 or BMP-9, with or without SWCNTs pre-treatment, in comparison to the CTL (p<0.05). At 48 h, in the absence of SWCNTs, the incubation with BMP-2 caused a reduction of approximately 15 % in proliferation relative to that of the CTL (p<0.05), while BMP-9 decreased the proliferation approximately 20 % (p<0.05). In the absence of BMPs, the cell proliferation was significantly decreased (approximately 30 %) in CNT in comparison with CTL after 48 h. Cells pre-treated with SWCNTs and then stimulated with BMP-2 or BMP-9 proliferated approximately 35 % less than CTL did at 48 h. However, the cells were able to significantly continue their growth between 24 and 48 h (p<0.05).

To determine whether the decreased proliferation observed within 48 h in the cells incubated with SWCNTs and stimulated with BMP-2 or BMP-9 was caused by apoptosis, a TUNEL assay was performed (Fig. 6.2B). Hoechst solution was used to visualise the nuclei of the cells. In the positive control cells, treatment with DNase I solution caused strand breaks in the DNA, and therefore the nuclei were clearly stained by the TUNEL reaction. In the CTL, as well as in cultures stimulated with BMP-2 or BMP-9 with or without SWCNT pre-treatment, only the Hoechst solution stained the nuclei, and no positive TUNEL cells were detected after 48 h, suggesting that the cells did not undergo apoptosis.

6.5.4 Differentiation of MC3T3-E1 preosteoblasts treated with SWCNTs and stimulated with BMP-2 or BMP-9

The decreased proliferation might be attributed to increased osteogenic differentiation [Luu et al., 2011]. Therefore, we measured the levels of osteogenic markers in MC3T3-E1 preosteoblasts stimulated with BMP-2 or BMP-9 in the presence or absence of SWCNTs (Figure 6.3).
Figure 6.2  Proliferation and apoptosis of MC3T3-E1 cells pre-treated with or without SWCNTs and stimulated with BMP-2 or BMP-9. (A) Proliferation of MC3T3-E1 cells pre-treated with or without SWCNTs for 4 h and then stimulated with BMP-2 or BMP-9 for 24 and 48 h, revealed using an MTS assay. The results are expressed as the mean ± SD of two independent experiments performed in triplicate. Statistical significant differences (p<0.05) from the CTL at 24 and 48 h are denoted by (*) and (+), respectively. (B) TUNEL assay performed on MC3T3-E1 cells incubated with or without SWCNTs for 4 h and then stimulated with BMP-2 or BMP-9 for 48 h. Bar = 100 μm. The results are representative of two independent experiments.

Early differentiation (Runx2, Dlx5, and Osx)

We investigated the early differentiation of MC3T3-E1 preosteoblasts incubated with or without SWCNTs and stimulated with BMP-2 or BMP-9 by evaluating transcripts encoding Runx2 (Fig. 6.3A) as well as Dlx5 (Fig. 6.3B) and Osx (Fig. 6.3C), which are downstream of Runx2 [Cohen, 2006].

At the 24 h time point, there was no significant difference between the Runx2 mRNA levels of CTL and CNT. Furthermore, adding BMP-2 or BMP-9 had no significant effect on the Runx2 transcript levels in the cells without SWCNT pre-treatment. Only the cells pre-treated with SWCNTs and stimulated with BMP-9 (CNT+BMP-9) showed a significant increase (p<0.05) in the Runx2 mRNA level over that of the CNT (Fig. 6.3A). At the 72 h time point, none of the conditions showed a significant increase in Runx2 transcript levels.
Similar Dlx5 mRNA levels were detected in CTL and CNT at 24 h. Moreover, stimulation of the cultures with BMP-2 or BMP-9, with or without SWCNTs pre-treatment, did not induce a significant augmentation of the Dlx5 transcript levels compared with those of CTL (Fig. 6.3B). After 72 h, however, only BMP-9 increased the amount of Dlx5 transcripts in comparison with those of CTL and CNT (p<0.05).

There was no significant difference in the Osx mRNA levels of CTL and CNT at both 24 and 72 h (Fig. 6.3C). However, stimulation with BMP-2 or BMP-9 increased the levels of Osx transcripts significantly (p<0.05) in both cultures with or without SWCNT pre-treatment after 24 h in the following order: BMP-9 > BMP-2 (p<0.05). At 72 h, the Osx transcript levels were not significantly increased in the cells incubated with BMP-2, with or without SWCNT pre-treatment. However, BMP-9 increased the Osx mRNA levels in cells, with or without SWCNT pre-treatment, significantly (p<0.05), in comparison with the level in CTL.

**Late differentiation (OC and ALP activity)**

The differentiation was further investigated by quantifying the OC mRNA levels (Fig. 6.4A) and measuring ALP activity (Fig. 6.4B). OC is the most abundant non-collagenous protein of the bone matrix and is a specific marker of mature osteoblasts [Senta et al., 2009]. ALP is also a non-collagenous protein secreted by osteoblasts that is essential for bone mineralisation and is a marker of osteoblast function [Havill et al., 2006].

At 24 h, no significant increase in the OC gene expression was detected in the CNT in comparison with the expression in CTL (Fig. 6.4A). Moreover, BMP-2 and BMP-9 did not induce an increase in OC mRNA levels in MC3T3-E1 preosteoblasts with or without SWCNT pre-treatment at 24 h. After 72 h, CNT still did not show any significant increase in OC transcripts compared with CTL. However, the cells stimulated with BMP-2 or BMP-9, with or without SWCNT pre-treatment, had significantly enhanced levels of OC mRNA (p<0.05) in comparison with CTL. Moreover, BMP-9 increased these transcripts more than BMP-2 did (p<0.05).

ALP mRNA was not detected in the CTL (Ct>30) or in the CNT (Ct>30). However, the ALP gene was slightly activated in the cells with or without SWCNT pre-treatment after incubation
with BMP-2 for 24 h (Ct=26.8 and 27.5, respectively) and 72 h (Ct=25.8 and 25.7, respectively) as well as in the cells that were pre-treated with or without SWCNTs and stimulated with BMP-9 for 24 h (Ct=25.9 and 26.4, respectively) and 72 h (Ct=25.5 and 24, respectively).

Figure 6.3 Early differentiation of MC3T3-E1 preosteoblasts incubated with or without SWCNTs and stimulated with BMP-2 or BMP-9. The levels of mRNA encoding the transcription factors Dlx5 (A), Runx2 (B) and Osx (C) in MC3T3-E1 cells incubated with or without SWCNTs for 24 h and stimulated with BMP-2 or BMP-9 for 24 and 72 h. The results are expressed as the mean ± SD of three independent experiments performed in triplicate. Statistically significant differences (p<0.05) from the CTL are denoted by a, from BMP-2 by b, from BMP-9 by c, from CNT by d, from CNT+BMP-2 by e, and from CNT+BMP-9 by f for each day.
The ALP activity was measured in the MC3T3-E1 preosteoblasts pre-treated with or without SWCNTs and stimulated with BMP-2 or BMP-9 to further verify the osteoblastic differentiation at the protein level (Fig. 6.4B). The CTL as well as the CNT had no ALP activity after 72 h. In contrast, significant ALP activity was detected in the cells stimulated with BMP-2 or BMP-9 for 72 h. Moreover, the incubation with BMP-9 resulted in higher ALP activity than the incubation with BMP-2 (p<0.05). However, in the presence of BMP-9, the MC3T3-E1 preosteoblasts pre-treated with SWCNTs showed less ALP activity than the cells without SWCNT pre-treatment did (p<0.05).

Figure 6.4 Late differentiation of MC3T3-E1 preosteoblasts incubated with or without SWCNTs and stimulated with BMP-2 or BMP-9. The level of mRNA encoding OC in MC3T3-E1 cells incubated with or without SWCNTs for 24 h and stimulated with BMP-2 or BMP-9 (1.92 nM) for 24 and 72 h. The results are expressed as the mean ± SD of three independent experiments performed in triplicate. Statistically significant differences (p<0.05) from the CTL are denoted by a, from BMP-2 by b, from BMP-9 by c, from CNT by d, from CNT+BMP-2 by e, and from CNT+BMP-9 by f for each day. (B) ALP activity was normalised to the total protein concentration. The results are expressed as the mean ± SD of two independent experiments performed in duplicate. Statistically significant differences (p<0.05) from the CTL are denoted by (*).
6.6 Discussion

In this study, we synthesised and purified SWCNTs that were produced using an induction thermal plasma process and verified their effect on bone-forming cells.

The purification procedure used in this study has been shown to be the most effective on the thermal plasma grown SWCNTs among other tested procedures which utilized a mixture of 1M HNO$_3$ and H$_2$O$_2$ or 6M HCl [Kim et al., 2009a]. Using TGA analysis, we verified that the selected purification protocol reduced the residual metal content to about 5 wt %, similar to our previously published results [Kim et al., 2009a]. High resolution scanning electron microscopy (HRSEM) and transmission electron microscopy (TEM) observations also confirmed the presence of SWCNTs bundles with ~10-20 nm thicknesses composed of individual SWCNTs in our sample similar to our previously published work [Kim et al., 2009a]. Furthermore, the presence of carboxylic groups (observed using XPS analysis) on the surface of the purified SWCNTs was in good agreement with other studies in which the SWCNT samples were purified using strong boiling nitric acid [Liu et al., 2010; Mu et al., 2009; Zhang and Yan, 2012].

Many studies have linked the toxicity of CNTs to the presence of metallic impurities in the samples [Alinejad et al., 2012; Banks et al., 2006; Kagan et al., 2006; Pumera and Iwai, 2009]. Although our purified SWCNTs still contained some metallic residues, the viability of MC3T3-E1 preosteoblasts was not affected after 24 h of incubation with the SWCNTs (0.1 to 100 µg/mL) (data not shown). This finding could be explained through a closer look at the suggested mechanism of CNT synthesis with high temperature processes such as thermal plasma which comprises melting of catalyst particles [Harutyunyan et al., 2005], dissolution of the carbon in liquid metals, and precipitation and growth of CNTs [Gorbunov et al., 2002]. Once the catalyst particle solidifies, a massive dissociation of the extra carbon dissolved in the particle results in the rapid encapsulation of the catalyst particle in a graphitic shell layer [Journet et al., 2012]. During the purification process, some of these graphitic shells break, and the trapped catalyst particles dissolve in the acid [Kim et al., 2009a]. In fact, empty graphitic shells have been observed using HRSEM in our purified SWCNT sample (data not...
shown). Sp² carbon is indeed a chemically inert material, and the only damage this carbon causes to the cells results from the mechanical disturbance [Pumera and Miyahara, 2009].

Moreover, the interaction between the materials and the attached cells can also be influenced by a complex biological system that involves protein adsorption [Li et al., 2012]. CNTs have been reported to favour cell function because of their ability to adsorb proteins from culture media [Aoki et al., 2007; Li et al., 2012]. In the present study, SWCNTs were suspended in cell culture medium supplemented with 10 % (v/v) FBS. Therefore, it may be possible that the adsorption of proteins from the FBS on the surface of SWCNTs formed a layer of biomolecules, influencing the observed cell behaviour. However, further experiments are required to characterise such protein adsorption using our experimental conditions.

Because activation of the BMP-induced Smad signalling pathway is involved in osteoblast function [Senta et al., 2009], a better understanding of the effect of CNTs on the ability of these bone-forming cells to respond to BMPs is crucial. Zhang et al. have recently shown that carboxylated MWCNTs (25 µg/mL) suppress BMP signalling in the mouse myoblast C2C12 cell line by binding to BMP type II receptors [Zhang et al., 2012]. In another study by this research team, carboxylated SWCNTs (100 µg/mL) caused the suppression of BMP signalling in human embryonic kidney epithelial (HEK293) cells [Mu et al., 2009] as well as C2C12 cell line [Zhang and Yan, 2012] and inhibited their proliferation (arresting the cell cycle at the G1/S transition phase). In contrast, we found that although the proliferation of MC3T3-E1 preosteoblasts was slightly affected by the purified SWCNTs, an accelerated phosphorylation of Smad1/5/8 was observed in the cultures pre-treated with these SWCNTs and incubated with BMP-2 or BMP-9. Moreover, the phosphorylated Smad1/5/8 translocated to the nuclei after 1 h. These findings suggested that our purified thermal plasma-grown SWCNTs, which had carboxylic groups on the surface, did not affect the ability of preosteoblasts to respond to BMP-2 and BMP-9 in terms of R-Smad phosphorylation and translocation. The discrepancies between our findings and the previous studies [Mu et al., 2009; Zhang et al., 2012; Zhang and Yan, 2012] may be explained by the differences between the cell types used and the CNT synthesis processes applied.
In addition, we found that stimulation with both BMP-2 and BMP-9 reduced the proliferation of MC3T3-E1 preosteoblasts without any increase in cell apoptosis. Several BMPs, including BMP-2 and BMP-9, induce osteogenic differentiation of bone cell precursors [Kang et al., 2004]. Moreover, proliferation and differentiation are inversely correlated, and there is a crucial balance regulation between these processes [Luu et al., 2011].

Our results showed that the SWCNTs did not interfere with the activation of the genes encoding proteins involved in the early and late osteoblastic differentiation induced by BMP-2 or BMP-9. Interestingly, a higher intensity of phosphorylated Smad1/5/8 was detected in the cells stimulated with BMP-2 compared with those stimulated with BMP-9 within 1 h, with or without SWCNT pre-treatment. However, BMP-9 seemed to induce osteoblastic differentiation more significantly than BMP-2 based on the Dlx5, Osx and OC mRNA levels. BMPs can also induce gene transcription through the mitogen-activated protein kinase (MAPK) pathway [Jun et al., 2010]. Our results suggest that pathways other than the Smad canonical pathway may also be involved in the induction of osteoblastic differentiation by BMP-9 in MC3T3-E1 preosteoblasts. Further experiments are therefore required to identify these pathways and study the effect of SWCNTs on them.

Carboxylated SWCNTs (30 µg/mL) down-regulate the expression of Runx2, ALP, and OC and inhibit the ALP activity of mouse MSCs in the presence of osteogenic media [Liu et al., 2010]. We found only a slight decrease in ALP activity in the cells pre-treated with SWCNTs and incubated with BMP-2 and BMP-9 in comparison with the cells stimulated with BMPs without SWCNT pre-treatment. However, evaluating the SWCNT effect on the ALP standard curve (data not shown) showed that SWCNTs adsorbed neither the p-Npp substrate nor the standard ALP provided in the commercial assay kit. Therefore, it appears that SWCNT pre-treatment may slightly affect the ability of MC3T3-E1 preosteoblasts to synthesise ALP protein. However, this effect was not drastic; the ALP activity of cells pre-treated with SWCNTs and incubated with BMP-2 or BMP-9 was significantly higher than that of the untreated cells.
6.7 Conclusion

In summary, considering that purified thermal plasma-grown SWCNTs induced cell proliferation reduction similar to BMP-2 and BMP-9 and did not affect the ability of preosteoblasts to respond to BMPs, they appear to have good potential to be used in bone applications. Purified SWCNTs could improve the properties of the scaffold without having negative effects on the differentiation and function of bone forming cells. Moreover, since BMP-9 showed a greater effect on the osteoblastic differentiation of MC3T3-E1 preosteoblasts than BMP-2 did, its use with SWCNTs appears to open promising avenues in the design of new biomimetic materials for bone regeneration.

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Dans cette thèse, la cytotoxicité et les effets cellulaires des matériaux présents dans les différentes étapes de la production des SWCNTs par RF-plasma thermique inductif (les matières premières commerciales utilisées comme catalyseurs et la source de carbone, les catalyseurs traités avec le procédé au plasma, le produit final contenant les SWCNTs, et les SWCNTs purifiés) ont été évalués. En outre, la capacité du système RF-plasma thermique inductif à produire des SWCNTs de haute qualité avec des matériaux non toxiques a été déterminée. Notre étude attire l'attention de l'industrie des nanomatériaux en particulier les producteurs de CNTs sur la nécessité de prendre en considération la toxicité des matériaux commerciaux utilisés pour la synthèse de CNTs. Ainsi, en comparant la cytotoxicité d'un groupe de catalyseurs métalliques (Co, Ni, Mo, et $Y_2O_3$), les particules de Co ont été identifiées comme étant les plus cytotoxiques. De plus, le procédé de RF-plasma thermique inductif peut affecter les propriétés physico-chimiques et la cytotoxicité des catalyseurs utilisés pour produire les CNTs. Par exemple, les particules de Ni se révèlent fortement cytotoxiques après le traitement par plasma, peut être en raison de la formation de sulfure de nickel.

D'autre part, cette étude montre que certains critères physico-chimiques tels la taille et la surface spécifique des particules ne peuvent pas expliquer à eux seuls la cytotoxicité observée avec les catalyseurs. De même, la libération des ions à partir des catalyseurs n'altère pas fortement la survie et la prolifération des cellules. Par contre, la composition des particules qui peut être modifiée par le procédé plasma semble expliquer la cytotoxicité des catalyseurs observée.

Étant donné que le catalyseur Co a montré une forte cytotoxicité, il était nécessaire de le remplacer par un autre catalyseur. Étant donné que les catalyseurs commerciaux Ni et Mo n'induisent pas une forte cytotoxicité, ils ont été utilisés comme substituts pour le Co comme matière première. L'effet des catalyseurs métalliques et de leur quantité sur la synthèse des
SWCNTs a été thermodynamiquement et expérimentalement étudié. Des SWCNTs de haute qualité peuvent être produits avec un mélange ternaire de catalyseurs contenant Ni, Co et Y₂O₃ ou avec un mélange binaire de Ni et Y₂O₃. Les calculs thermodynamiques indiquent que le type et la quantité des catalyseurs métalliques utilisés pour la production des SWCNTs dans le système réactionnel de RF-plasma thermique inductif affectent plus la phase en solution liquide que la phase gazeuse. En considérant la température eutectique du système de catalyseur métallique-carbone, le temps de séjour effectif dans le RF-plasma thermique inductif du Ni / Co a été déterminé comme étant 2,3 fois plus important que celui du Mo ce qui accroît la qualité des SWCNTs produits. Ainsi, le potentiel du Mo pour la synthèse de SWCNT peut être amélioré par l'optimisation du procédé de RF-plasma thermique inductif en augmentant la longueur de la zone à haute température (T> 2 000 K).

Les SWCNTs qui ont été synthétisés en utilisant divers mélanges de catalyseurs (Ni-Co-Y₂O₃, Ni-Y₂O₃, et Ni-Mo-Y₂O₃) n'ont pas montré de différence significative de leur cytotoxicité. De plus, la procédure utilisée pour le traitement des cellules avec les SWCNTs peut affecter les résultats. La perte de viabilité cellulaire causée par la perturbation mécanique de la membrane plasmique ne doit pas être confondue avec la cytotoxicité intrinsèque des SWCNTs.

En négligeant la perte de viabilité causée par la perturbation mécanique de la membrane plasmique, les SWCNTs produits par RF-plasma thermique inductif n'ont pas fortement affecté la viabilité ou la prolifération des préostéoblastes MC3T3-E1. Bien que des expériences complémentaires restent à faire pour obtenir de nouvelles informations sur le profil toxicologique de ces SWCNTs, il peut être suggéré que les SWCNTs produits par RF-plasma thermique inductif sont prometteurs en application osseuse.

Ainsi, étant donné que les SWCNTs synthétisés par plasma thermique et purifiés, n'ont pas réduit la viabilité des préostéoblastes ni affectés leur capacité à répondre aux BMPs, ils semblent avoir un bon potentiel pour être utilisés dans les comblements osseux. Ils pourraient améliorer les propriétés des substituts osseux sans avoir d'impacts négatifs sur la fonction et la différenciation des cellules. En fait, l'activation de la voie de signalisation des Smad par les BMPs a été accélérée lors du prétraitement des préostéoblastes MC3T3-E1 par les SWCNTs. De plus, les SWCNTs n'ont pas affecté la capacité de ces cellules à se différencier sous
l'influence de la BMP-2 ou BMP-9. En outre, la BMP-9 a montré un plus grand effet sur la différenciation ostéoblastique des préostéoblastes MC3T3-E1 par rapport à la BMP-2. La combinaison de la BMP-9 avec les SWCNTs semble donc ouvrir des perspectives prometteuses dans la conception de nouveaux matériaux biomimétiques pour la régénération osseuse.

7.2 Global conclusion

In this PhD project, fields of nanomaterial synthesis, biology and toxicology assessments were combined to provide the opportunity to improve the production of SWCNTs by RF induction thermal plasma for their ultimate applications in bone related biomaterials systematically and safety-wise.

In this regard, the cytotoxicity of different materials present in various steps of SWCNT production by RF induction thermal plasma (i.e. the commercial raw materials used as catalysts and carbon source, the particles treated with the plasma process, the SWCNT product, and the purified SWCNTs) were evaluated.

Our study drew attention of nanomaterial industry particularly CNT producers to consider the toxicity of feedstock materials. Indeed, comparison of the cytotoxicity of a group of popular metallic catalysts used for CNT production showed that Co particles were the most cytotoxic catalyst among Co, Ni, Mo, and Y₂O₃. Moreover, it was shown that the process that is used for CNT synthesis can change the physico-chemical properties of the feedstock materials and subsequently their cytotoxic effects on the cells. For example, Ni particles which did not show strong cytotoxicity in the commercial form became strongly cytotoxic after being treated with the RF induction thermal plasma. It was also shown that particle size and surface area cannot fully explain the observed cytotoxicity of the catalysts. Chemical composition of particles that can be modified during the synthesis process may be the most reliable property to justify catalyst cytotoxicity. Moreover, since the release of ions from the catalysts did not strongly impair proliferation and survival of the cells, direct contact with the cells seems to be an important parameter for causing cytotoxicity by catalyst particles.
Given that Co showed strong cytotoxicity, replacing it by a non-toxic catalyst could reduce health risks of SWCNT production by RF induction thermal plasma process. Since commercial Ni and Mo did not show strong cytotoxicity, they were used as substitutes for Co in the feedstock material for SWCNT synthesis. The experimental results showed that catalyst type affected the quality of SWCNT final product. Similar quality SWCNTs were produced when the same amount of Co was replaced by Ni in the catalyst mixture. However, the quality of the SWCNTs produced from the feedstock material that contained Mo was lower than that synthesized from mixtures containing Ni and Co. Taking in to account the eutectic temperature of the catalyst-carbon system, the effective residence time for Ni/Co was found to be 2.3 times more than that of Mo in the RF induction thermal plasma. Consequently, higher quality SWCNTs were synthesized from Ni and Co compared to Mo. Therefore, optimizing the operating conditions of the RF induction thermal plasma process for producing high quality SWCNTs using Mo is necessary.

SWCNT samples which were synthesized using different catalyst mixtures (i.e. Ni-Co-Y_2O_3, Ni-Y_2O_3, and Ni-Mo-Y_2O_3) did not show significant cytotoxicity differences. Indeed, the RF induction thermal plasma grown SWCNTs neither strongly affected the viability of MC3T3-E1 preosteoblasts seeded on these materials nor their proliferation.

Moreover, it was shown that the procedure employed for treating cells with SWCNTs can affect the results of cytotoxicity assessment and even can mislead experimenters with false conclusions. In other words, in *in vitro* experiments, the methodology by which cells are being exposed to materials may influence the obtained results. Cell damages caused by the exposure conditions should not be mistaken for the intrinsic cytotoxicity of the materials. Our results showed that SWCNTs suspended in cell culture medium could cause loss of viability in cells mainly as a result of mechanical disturbance. However, when SWCNTs were used as substrates, on which cells were seeded, they did not cause significant cell viability loss.

The SWCNTs were purified and their effect on the proliferation, differentiation, and function of bone forming cells were investigated under the influence of BMPs. It was shown that the purified thermal plasma grown SWCNTs neither reduced the viability of preosteoblasts nor
affected their ability to respond to BMPs. Therefore, they are introduced as nanomaterials with promising potential to be used in bone applications because they could improve the properties of the biomaterials without having negative impacts on the function and differentiation of cells. In fact, pre-treatment with SWCNTs accelerated the BMP induced Smad signaling pathway in MC3T3-E1 preosteoblasts and did not affect the ability of these cells to differentiate under the influence of BMP-2 or BMP-9. Moreover, BMP-9 showed a greater impact on osteoblastic differentiation of MC3T3-E1 preosteoblasts compared to BMP-2 in cells pretreated with and without SWCNTs. Therefore, combination of BMP-9 with SWCNTs seems to open promising avenues in the design of new biomimetic materials for bone regeneration.

7.3 Perspectives

- **Optimizing the process for synthesis of SWCNTs with Mo:** It was observed that Mo catalyst has the potential to produce SWCNT using RF induction thermal plasma method. However, the present optimized operating conditions are not quite suitable for this catalyst. Therefore it is necessary to increase the effective residence time of Mo catalyst in the reactor. This can be achieved by changing the operating conditions such as increasing the power plate, changing the plasma gas composition and/or modifying the shape, thickness or thermal property of the graphite insert in the reactor.

- **Investigating the mechanism by which SWCNTs may stimulate oxidative stress or genotoxicity:** Nanoparticles may cause genotoxicity by entering into the nucleus of the cells and interacting directly with the DNA or through the oxidative attack by inducing oxidants such as reactive oxygen species (ROS) which result in impaired antioxidant defense and oxidative stress [Donaldson et al., 2010b]. Therefore, it is interesting to study the possibility of the catalysts and/or SWCNTs uptake by the cells using HRTEM and measure the production of reactive species and inflammatory molecules after exposure to SWCNTs for longer times (e.g. 7 days).

- **Evaluating other assays for SWCNT cytotoxicity testing:** Since our results showed a clear interaction of neutral red assay with SWCNTs and therefore non-reproducible results with this assay, it can be interesting to evaluate other assays which can determine the
cytotoxicity of SWCNTs in order to find another reliable method for investigating the cellular effects of these nanomaterials.

- **Studying the effect of SWCNTs on the ALP protein synthesis:** Incubation with SWCNTs did not affect the ability of MC3T3-E1 preosteoblast to differentiate and express genes encoding different osteogenic markers. Indeed, PCR analyses showed that cells incubated with SWCNTs for 24 h and stimulated with BMP-2 or BMP-9 were perfectly able to function in the level of gene expression and creating mRNA to pass the message for protein synthesis. However, our results showed that these cells were not as efficient as untreated cells in ALP protein synthesis. Therefore, in the next step, it is necessary to understand which step of protein synthesis (after mRNA release from the nucleus) is affected by these SWCNTs.

- **Studying the effect of SWCNT on the mineralization:** Effect of SWCNTs on the ability of preosteoblasts to fully differentiate and become mature osteoblasts capable of performing their very important responsibility which is mineralization of the bone matrix should be studied by labeling the Ca^{2+} deposits using Alazarin red.
# APPENDIX A

## INCORPORATION OF CNTS IN COMPOSITES MADE OF SYNTHETIC POLYMERS

<table>
<thead>
<tr>
<th>CNT</th>
<th>Matrix</th>
<th>Cell/animal</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCNTs</td>
<td>PMMA</td>
<td>-</td>
<td>- Increase in Young’s modulus and fracture toughness of PMMA when reinforced with MWCNTs</td>
<td>[Marrs et al., 2006]</td>
</tr>
<tr>
<td>SWCNTs</td>
<td>Poly(propylene fumarate) (PPF)</td>
<td>Rat bone marrow stromal cells (MSCs)</td>
<td>- Significant reinforcement of the PPF by SWCNTs - Good attachment and proliferation of the cells on the scaffolds</td>
<td>[Shi et al., 2007]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Polycarboxislane (PCS)</td>
<td>Wister-strain rats</td>
<td>- Good osteoconductivity of the MWCNT/PCS composite (formation of the new bone tissue in the femur with a little inflammatory response in the subcutaneous tissue)</td>
<td>[Wang et al., 2007]</td>
</tr>
<tr>
<td>SWCNTs</td>
<td>PPF and propylene fumarate diacrylate (PF-DA)</td>
<td>New Zealand white rabbits</td>
<td>- Favorable hard and soft tissue responses of the nanocomposite scaffolds at 4 and 12 weeks after implantation - A greater bone tissue in growth with SWCNT scaffolds compared to control polymer scaffolds at 12 weeks</td>
<td>[Sitharaman et al., 2008]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Polyurethane (PU)</td>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>- Improvement of the compression strength and stiffness of the scaffold by MWCNTs - No cytotoxicity or detrimental effects on osteoblast differentiation or mineralization by MWCNT loading</td>
<td>[Stevens et al., 2008]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>PU</td>
<td>Human osteosarcoma cell line (Saos-2)</td>
<td>- Improved the wettability of the nanocomposite with CNT incorporation. - No cytotoxicity or detrimental effect on the differentiation or mineralization of the cells with MWCNT loading</td>
<td>[Verdejo et al., 2009]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Poly(L-lactic acid) (PLLA)</td>
<td>Human fetal osteoblasts (hFOB 1.19)</td>
<td>- Higher stiffness and higher cell viability in the 3D microfabricated PLLA/MWCNTs nanocomposite scaffolds than the pure 3D microfabricated PLLA scaffolds</td>
<td>[Vozzi et al., 2012]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Poly(lactic-co-glycolic acid) (PLGA)</td>
<td>Osteoblasts</td>
<td>- Increased cell proliferation and modulated osteogenic differentiation, well-defined stress fibers and enhanced phosphorylation of focal adhesion kinase (FAK) in osteoblasts cultured on the PLGA-MWCNTs films. - ERK1/2 were selectively phosphorylated in osteoblasts grown on PLGA-MWCNTs film.</td>
<td>[Jiang et al., 2012]</td>
</tr>
</tbody>
</table>
# APPENDIX B

## INCORPORATION OF CNTS IN COMPOSITES MADE OF NATURAL POLYMERS

<table>
<thead>
<tr>
<th>CNT</th>
<th>Matrix</th>
<th>Cell/animal</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| SWCNTs  | Collagen| Rat aortic smooth muscle cells (RASMC)            | - Composites with high mechanical strength  
- More than 85% cell viability after 3 and 7 days                                          | [MacDonald et al., 2005]           |
| MWCNTs  | Collagen| -                                               | - Improvement of thermal stability, infrared emissivity and mechanical properties         | [Cao et al., 2007]          |
| MWCNTs  | Collagen| Murine preosteoblasts (MC3T3-E1)                 | - Slightly lower cell viability and proliferation but higher cell adhesion on the MWCNT-coated dishes than on the collagen-coated dishes | [Terada et al., 2009]        |
| MWCNTs  | Collagen| Murine preosteoblast (MC3T3-E1)                 | - Higher cell attachment and proliferation with MWCNT coating to the collagen sponge    | [Hirata et al., 2009]       |
| SWCNTs  | Collagen| Pulmonary arterial endothelial cells             | - High viability and adhesion of cells at 1 wt % SWCNTs loading concentration             | [Tan et al., 2010]          |
| MWCNTs  | Collagen| Human osteosarcoma cell line (Saos-2)            | - Improved the cell adhesion with MWCNT coating on the inside of the collagen sponge    | [Hirata et al., 2010]       |
| MWCNTs  | Collagen| Rat primary osteoblasts (ROBs) and Wistar rats   | - Earlier differentiation of the cells on the MWCNT-coated sponge than on the uncoated sponge  
- More extensive amounts of new bone formation around MWCNT-coated sponges than those around uncoated sponges at 28 and 56 days | [Hirata et al., 2011]           |
| MWCNTs  | Chitosan| Mouse myoblastic cells (C2C12) and Balb type mice | - Biocompatibility of the MWCNT/chitosan scaffolds in terms of cell adhesion, spreading, proliferation and viability  
- Absence of chronic inflammation and bone tissue regeneration after 3 weeks | [Abarrategi et al., 2008]       |
| SWCNTs  | Chitosan| Human fetal osteoblasts (hFOB 1.19)              | - High osteoblast density with chitosan nanocomposite containing SWCNTs                   | [Im et al., 2012]          |
| SWCNTs  | Hyaluronic acid | -                                              | - Enhancement of the dynamic mechanical properties of the hybrid hydrogels compared to the native ones | [Bhattacharyya et al., 2008] |
## INCORPORATION OF CNTS IN COMPOSITES MADE OF HYDROXYAPATITE

<table>
<thead>
<tr>
<th>CNT</th>
<th>Cell/animal</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCNTs</td>
<td>-</td>
<td>- 38% higher strength of the HAP/MWCNT composite compared to single phase HAP</td>
<td>[Zhao and Gao, 2004]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>-</td>
<td>- Massive growth of needle shaped Ca-P crystallites at nanoscale levels on MWCNTs.</td>
<td>[Akasaka et al., 2006; Akasaka and Watari, 2005]</td>
</tr>
<tr>
<td>SWCNTs</td>
<td>-</td>
<td>- Nucleation and crystallization of HAP on the SWCNTs</td>
<td>[Zhao et al., 2005]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>-</td>
<td>- Increased strength of HAP by MWCNTs without reduction of the bioactivity</td>
<td>[Chen et al., 2006]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>-</td>
<td>- Nucleation of the HAP crystals form simulated body fluid within 7 days by MWCNTs and formation of hierarchy assemblies</td>
<td>[Aryal et al., 2006b; Aryal et al., 2006a]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Mature big white rats</td>
<td>- Higher bending strength and fracture toughness of MWCNTs/HAP composite compared to those of pure HAP - No serious inflammation reaction with MWCNTs/HAP composite embedded into the striated muscle of the mouse</td>
<td>[Li et al., 2007]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Human osteoblasts</td>
<td>- Promotion of the fracture toughness and crystallization of the composite with MWCNTs reinforced HAP coating - A positive effect on the spreading and phenotype maturation of the cells</td>
<td>[Balani et al., 2007]</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>-</td>
<td>- Bone like apatite formation in the matrix of the HAP/MWCNTs composites when incubated in simulated body fluid</td>
<td>[Niu et al., 2010]</td>
</tr>
</tbody>
</table>
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analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. *Bioorganic and Medicinal Chemistry Letters*, volume 1, number 11, p. 611-614.


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